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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: TOXIN GENE FROM XENORHABDUS NEMATOPHILUS

(57) Abstract

Purified insecticidal toxins and biologically active fragments thereof, and polynucleotide molecules encoding same, from the bacteria *Xenorhabdus nematophilus* are described.

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TOXIN GENE FROM XENORHABDUS NEMATOPHILUS

Technical Field

The present invention concerns the identification and isolation of a new class of protein toxins specific against insects which are produced by bacteria from the species *Xenorhabdus nematophilus* and possibly by the species *X.beddingii*. In addition, the present invention relates to the insertion of this class of toxin into recombinant viruses, bacteria, protozoa, fungi, and transgenic plants in order to broaden the use of these toxins for control of a large range of insect pests and plant parasitic nematodes.

Background

Insect pathogenic nematodes of the family *Steinernematidae* are known to be symbiotically associated with bacteria of the genus *Xenorhabdus*. It has been observed that these bacteria have the ability to kill a wide range of different insects without the aid of their nematode partners.

The present inventors have identified a new class of toxins. A DNA fragment encoding one of these toxins has been isolated from *Xenorhabdus nematophilus* stain A24 and characterised by sequencing. As will be recogised by persons skilled in the art, DNA fragments encoding members of this new class of toxins may be usefully introduced into viral agents, including entomopox and nuclear polyhedrosis viruses; bacteria (including *Gracilicutes, Firmicutes, Tenericutes* and *Mendosicutes*); fungi; protozoa; and plants.

Summary of the Present Invention

In a first aspect, the present invention consists in a polynucleotide molecule comprising a nucleotide sequence which encodes an insecticidal toxin and which is at least 70% homologous to the nucleotide sequence shown in Table 1 from residue 83 to 919, or a fragment thereof which fragment encodes an insecticidal polypeptide.

In a preferred embodiment of the present invention the nucleotide sequence is at least 90% to the sequence shown in Table 1 from residue 83 to 919.

Preferably, the nucleotide sequence which encodes an insecticidal toxin from *Xenorhabdus* and more preferably, the nucleotide sequence substantially corresponds to the sequence shown in Table 1 from residue 83 to 919.

In a second aspect the present invention provides in an insecticidal toxin which includes an amino acid sequence which is at least 70% homologous to residues 1 to 278 shown in Table 2 or a functional fragment thereof.

In a preferred embodiment of the present invention the insecticidal toxin includes an amino acid sequence which is at least 90% homologous to residues 1 to 278 shown in Table 1 or a functional fragment thereof.

In a further preferred embodiment the insecticidal toxin includes an amino acid sequence substantially corresponding to residues 1 to 278 in Table 1 or a functional fragment thereof.

In a third aspect the present invention provides in a recombinant organism, the organism being characterised in that it is transformed with the polynucleotide molecule of the first aspect of the present invention.

The organisms which may be usefully transformed with the polynucleotide molecule of the first aspect of the present invention include viral agents such as entomopox and nuclear polyhedrosis viruses; bacteria, such as *Gracilicutes, Firmicutes, Tenericutes* and *Mendosicutes;* fungi; protozoa; and plants.

The term "substantially corresponds" as used herein in relation to the nucleotide sequence is intended to encompass minor variations in the nucleotide sequence which due to degeneracy do not result in a change in the encoded protein. Further this term is intended to encompass other minor variations in the sequence which may be required to enhance expression in a particular system

but in which the variations do not result in a decrease in biological activity of the encoded protein.

The term "substantially corresponding" is used herein in relation to the amino acid sequence is intended to encompass minor variations in the amino acid sequence which do not result in a decrease in biological activity of the insecticidal toxin. These variations may include conservative amino acid substitutions. The substitutions envisaged are:-

G, A, V, I, L, M; D, E; N, Q; S, T; K, R, H; F, Y, W, H; and P, Nα-alkalamino acids.

As used herein the term "functional fragments" is intended to encompass fragments of the insecticidal toxin which retain insecticidal activity.

In a fourth aspect, the present invention provides a method for controlling the proliferation of insects, comprising applying to an infested area a recombinant organism according to the third aspect optionally in admixture with an acceptable agricultural carrier.

Isolation and Characterisation of a Toxin from *Xenorhabdus nematophilus* A24

Generation of a Cosmid Library

Genomic DNA from *Xenorhabdus nematophilus* A24, isolated using the method of Marmur (1961) was partially digested using the restriction enzyme Sau 3A, to generate fragments of DNA that were in the size range of 30 to 50 kilobasepairs (kb), and dephosphorylated using the enzyme calf alkaline phosphatase. The cosmid "Supercos" (Stratagene) was prepared to receive foreign insert DNA into its Bam HI cloning site according to the manufacturer's instructions. The digested DNA from *X.nematophilus* A24 was added to the cosmid DNA in a ratio of 3:1 and ligated together using the enzyme T4 DNA ligase. The ligated material was subsequently packaged into λ-bacteriophage using the Gigapack II XL Packaging Extract (Stratagene) as per the manufacturer's

instructions. The packaged DNA was subsequently transfected into the *Escherichia coli* strain NM554 (F-, recA, araD139,Δ (ara, leu) 7696, Δlac Y74, galU-, galK-, hsr, hsm^+, strA, mcrA[-], mcrB[-]. Bacteria were plated out onto Luria Bertani (LB) agar plates containing 150 μg ml^{-1} ampicillin to select for those bacteria containing recombinant Supercos plasmids.

Screening for Toxin Producing Clones

Individual clones were grown overnight at 28°C in LB containing 150 μg ml^{-1} ampicillin. Cultures were treated for 15 minutes with 2mg ml^{-1} lysozyme in order to release any proteins produced by the recombinant DNA into the medium. Five μl aliquots of this solution were then injected directly into the haemocoel of three *Galleria mellonella* fourth instar larvae. Appropriate controls containing lysozyme and non-recombinant *E.coli* NM554 cultures were also injected to confirm the absence of any toxicity to these larvae. Two clones were found to have strong insecticidal activity. Injected larvae were found to be very sluggish after 30 hours, with all larvae dead within three days.

Characterisation of Toxin Producing Clones

The recombinant Supercos DNA from these clones was isolated using an alkaline lysis procedure (Maniatis et al., 1982). Isolated DNA was digested with varying restriction enzymes and analysed using TAE agarose gel electrophoresis (Maniatis et al, 1982). It was found that both clones were identical and contained a 34.6 kb DNA insert from *X. nematophilus* A24. One of these clones cos149 was chosen for further study.

A 7.4kb Bam HI fragment from cos149 was cloned into the plasmid vector pGEM7Z(f)+ (Promega) which was transformed into the *E.coli* strain DH5α (F-, Φ80dlac ZΔ M15, recA1, endA1, gyrA96, thi-1, hsdR17[r_K-, m_K+] sup E44, relA1, deoR, Δ[lacZYA-argF] U169) using electroporation at 25μF, 200Ω and 2.5kV in a 0.2cm

cuvette in a Bio-Rad Gene Pulser. This clone (N8pGEM) was found to continue to be toxic against *G.mellonella* larvae.

Plasmid DNA from N8pGEM was isolated and digested with the restriction enzymes ClaI and SphI. This resulted in the linearization of this plasmid containing one end (3') which was resistant to digestion by the enzyme Exonuclease III and the other end (5') which could be digested at a constant rate of 450 bases per minute at 37°C by this enzyme using the Erase-a-Base kit from Promega. Using this enzyme aliquots containing decreasing size plasmids were obtained which were recircularised using the enzyme T4 DNA ligase. Recircularised plasmids were reintroduced into the bacterium *E.coli* strain DH5a using electroporation (see above). Varying size clones were selected and used for injecting *G.mellonella* larvae. The smallest clone which continued to be insecticidal was found to contain 1.5kb of *X.nematophilus* A24 DNA and was designated tox 1.

Plasmid DNA from tox 1 was isolated and digested with the restriction enzymes Sac I and HindIII, respectively to again create linear molecules with one end resistant and the other sensitive to digestion with Exonuclease III. Deletion mutants were isolated and tested against *G.mellonella* larvae. A clone which now only contained 1.2kb of *X.nematophilus* A24 DNα was isolated and was toxic against our test insect. This clone was designated toxb4.

The recombinant plasmids from toxb4 and three further (non-toxic) deletion clones, toxb5, toxb6 and toxb7, were isolated and used for obtaining the sequence of both strands of the toxin gene. Sequencing was performed using the Applied Biosystems, Incorporated Model 370 automated sequencer. Sequencing templates were prepared using double stranded DNA templates and the 21M13 and SP6 primer sites located on the pGEM7Z(f)+ plasmid and

using the Taq dye primer cycle sequencing protocol (Applied Biosystems, Incorporated).

The toxin gene was found to consist of an 834 basepair open reading frame (Table 1) which translates into a 278 amino acid protein (Table 2). The start of the toxin gene sequence was preceded by appropriate DNA promoters necessary for transcription of the gene into a mRNA molecule prior to its synthesis into a peptide. These consist of a Shine-Dalgarno poly-purine sequence and -10 and -35 RNA polymerase recognition sequences (Table 1).

The DNA sequence and the derived amino acid sequences were analysed by sequence data bank analyses to determine if any other related sequences have previously been identified. The results indicated that no other sequence exists in the GenBank and EMBL data banks which has any similarity to this gene and its product.

Cloning of Xenorhabdus Toxin into a High-Expression Vector

Using the determined DNA sequence, 20-mer DNA primers were designed to cover the 5' and 3' region of the toxin gene and thus allow PCR amplification of the toxin and subsequent insertion into an expression vector. These primers included linker regions containing appropriate restriction enzyme sites (ClaI and NdeI for the 5' primer and Bam HI for the 3' primer).

5' primer CCATCGATCATATGGTTATTAAACC

3' primer CGGGATCCTTATCTCTAAGGTTTTT

Utilising a standard PCR protocol (Innis, M.A., Gelford, D.H., Sminsky, J.J. and White, T.J.: (1990). PCR Protocols : A Guide to Methods and Applications. Academic Press, San Diego. 482pp) the toxin was amplified out of the genome of *X.nematophilus* A24 and restriction digested with Cla I and Bam HI. The digested fragment was subsequently ligated into pGEM-7Zf(+) and then subcloned from this vector into the high expression vector pT7T2b(derived from pET11 [Novagen] and carrying the T7

promoter upstream from the start of the toxin insert; constructed by Dr. Karl Gordon, CSIRO, Division of Entomology) using the restriction enzyme sites Nde I and Bam HI. The recombinant plasmid was transformed into the E. coli strain BL21(DE3)[F-ompT r_B -m_B -, which carries in its chromosome the T7 RNA polymerase gene under lac UV5 control). Induction of the toxin may be achieved by the addition of 0.4mM IPTG at mid-exponential phase of the culture and continuing the incubation for an extra 4 hours.

In vitro expression of the 1.2 Kb insert fragment from toxb4 was achieved with the *E.coli* S30 Extract Procaryotic Translation System for linear DNA. Only a 30kDa peptide was produced indicating that the 1.2 Kb fragment encodes one peptide only - the insect toxin.

Southern Blot Hybridization of a Range of Xenorhabdus spp. and Photohabdus Luminescens Strains with the X. nematophilus A24 Toxin Gene

DNA isolated from a range of *Xenorhabdus* species and *Photohabdus* (bacteria symbiotically associated with nematodes from the family *Heterohabditidae*) controls was digested to completion with the restriction enzyme Eco RV and run out on a 0.8% TAE agarose gel and the DNA fragments blotted and fixed onto a Hybond-N+ membrane (Amersham) as per the manufacturer's instructions.

The toxin gene was radiolabelled with ^{32}P using nick translation (Maniatis *et al.*, 1982) and probed against the blot containing the DNA of a range of *Xenorhabdus* and *Photohabdus* strains (Maniatis *et al.*, 1982). Under moderate stringency wash conditions at 65°C(0.1% SDS, 1% SSPE, Maniatis *et al*, 1982) the toxin only hybridised to *X. nematophilus* and *X. beddingii* strains. However, the toxin gene did not show any homology to the DNA from strains of *X. bovienii*, *X. poinarii*, some unclassified *Xenorhabdus spp.* and *Photohabdus luminescens*. This result suggests that this toxin type is confined to strains from

the species *X. nematophilus* and *X. beddingii*. As *X. beddingii* has insecticidal activity and shows homology to the toxin gene it is most probable that these sequences are part of related/similar yet slightly different toxins. A high stringency wash at 65°C(0.1% SDS, 0.1% SSPE; Maniatis *et al.* 1982) of the blot removed the message from the *X.beddingii* strain, but not from the *X.nematophilus* strains.

Characteristics of the Toxic Protein Product

The toxin is inactivated by heating to 65°C for 15 minutes, yet stable at 45°C. Sodium dodecyl sulphate at a concentration of 0.1% does not inactivate this toxin thereby indicating extreme stability and thereby a protein which will fold into its appropriate form under a wide range of different conditions (which includes most cell types).

This new class of toxin may be purified by one or more methods of protein purification well known in the art. Insecticidal fragments may be generated from the purified toxin using, for example, cleavage with trypsin or cyanogen bromide.

As will be appreciated by those skilled in this field, the present invention provides a new class of toxins useful for genetically engineering a wide range of biological systems which will thus become more useful for control of insect pests detrimental to agricultural, aquatic and forest industries.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

TABLE 1

```
   1  AAGAAACCGT AACAGCGGAA ATCAACGCTG CAATTTATAT TAGTAGTCAT
                                      Start          -35
  51  TTCAATAAAC GCCAACATAA TGGGAAAGTA CAATGGTTAT TAAACCCGTA
                      -10      S-D
 101  ACAACTCCGA GTGTAATACA ATTAACGCCT GATGATAGAG TAACGCCTGA
 151  TGATAAAGGT GAATATCAAC CCGTTGAAAA GCAAATAGCG GGAGATATAA
 201  TACGTGTACT AGAATTCAAG CAAACAAATG AAAGTCATAC AGGATTGTAT
 251  GGAATTCCAT ATCGAGCTAA GAAAGTAATA ATAGCATATG CTTTAGCGGT
 301  AAGTGGTATT CATAATGTCT CTCAACTTCC AGAAGACTAT TATAAAAATA
 351  AGGATAACAC AGGTAGAATT TATCAAGTAT ACATGTCTAA TCTTTTATCT
 401  GCACTATTGG GTGAGAATGG TGATCAAATT TCTAAAGATA TGGCAAATGA
 451  TTTTACCCAG AACGAACTGG AGTTTGAGGT CAACGTCTTA AAAATACCTG
 501  GGATATTCCT GATCTTGAGA ATAAACTATT GGAAGATTTA TTCAGATGAA
 551  GATAAATTAT TAGCACTATA TTTCTTTGCT TCACAAGAAC TTCCAATGGA
 601  GGCAAATCAA CAATCAAATG CAGCAAATTT TTTTAAAGTA ATTGATTTTT
 651  TACTTATCTT ATCTGCTGTA ACATCACTGG GAAAAAGGAT TTTTTCAAAA
 701  AATTTTTACA ATGGTCTAGA AACTAAATCA TTAGAGAATT ATATTGAGAG
 751  AAAAAAACTT TCTAAACCTT TCTTTCGACC ACCGCAGAAG TTACCTGATG
 801  GCAGAACAGG CTACTTGGCC GGTCCAACAA AAGCGCCTAA ATTGCCAACA
 851  ACGTCTTCTA CAGCAACAAC GTCTACAGCA GCTTCATCTA ATTGGAGAGT
 901  TAGTTTGCAA AAACCTTAGA GATAACCCAT CCAGAAATAC ATTTATGAAA
                    Stop
 951  ATGGATGATG CTGCAAAACG AAAATATAGT TCATTTATAA AAGAGGTACA
1001  AAAGGGTAAT GATCCACGTG CAGCAGCAGC AAGTATTGGT ACAAAAAGCG
1051  GCAGTAACTT CGAAAAACTG CAAGGTAGAG ATTTATATAG TATAAGACTA
1101  AGCCAAGAAC ACAGGGTAAC ATTCTCCATA AATAATACTG ACCAAATAAT
1151  GGAGATCCAA AGTGTTGGAA CTCATTACCA AAATATATAA CCTGATTTAT
1201  AGTAGTGATA AGACGTAAGA TAAATATGGA AGGTTGTAAT TCTATTGCAC
1251  TTCCTCAGAG GTGACCGCTC AG
```

TABLE 2

```
  1  MVIKPVTTPS VIQLTPDDRV TPDDKGEYQP VEKQIAGDII RVLEFKQTNE
 51  SHTGLYGIPY RAKKVIIAYA LAVSGIHNVS QLPEDYYKNK DNTGRIYQVY
101  MSNLLSALLG ENGDQISKDM ANDFTQNELE FEVNVLKIPG IFLILRINYW
151  KIYSDEDKLL ALYFFASQEL PMEANQQSNA ANFFKVIDFL LILSAVTSLG
201  KRIFSKNFYN GLETKSLENY IERKKLSKPF FRPPQKLPDG RTGYLAGPTK
251  APKLPTTSST ATTSTAASSN WRVSLQKP*R *PIQKYIYEN G*CCKTKI*F
301  IYKRGTKG** STCSSSKYWY KKRQ*LRKTA R*RFI*YKTK PRTQGNILHK
351  *Y*PNNGDPK CWNSLPKYIT *FIVVIRRKI NMEGCNSIAL PQR*PL
```

CLAIMS:

1. A polynucleotide molecule comprising a nucleotide sequence which encodes an insecticidal toxin and which is at least 70% homologous to the nucleotide sequence shown in Table 1 from residue 83 to 919, or a fragment thereof which fragment encodes an insecticidal polypeptide.

2. A polynucleotide molecule as claimed in claim 1 in which the nucleotide sequence is at least 90% homologous to the nucleotide sequence shown in Table 1 from residue 83 to 919.

3. A polynucleotide molecule comprising a nucleotide sequence substantially corresponding to the sequence shown in Table 1 from residue 83 to 919 or a fragment thereof, which fragment encodes an insecticidal polypeptide.

4. A polynucleotide molecule according to claim 7, wherein the nucleotide sequence encodes an insecticidal toxin, or an insecticidal fragment thereof, from *Xenorhabdus nematophilus*

5. A polynucleotide nucleotide molecule according to any one of claims 1 to 4 in which the molecule is a DNA molecule.

6. A purified insecticidal toxin, or functional fragment thereof, from the bacterial genus *Xenorhabdus*.

7. A purified insecticidal toxin, or functional fragment thereof, from *Xenorhabdus nematophilus*.

8. An insecticidal toxin which includes an amino acid sequence which is at least 70% homologous to residues 1 to 278 shown in Table 2 or a functional fragment thereof.

9. An insecticidal toxin as claimed in claim 8 in which the toxin includes an amino acid sequence which is at least 90% homologous to residues 1 to 278 shown in Table 2 or a functional fragment thereof.

10. An insecticidal toxin, the toxin including an amino acid sequence substantially corresponding to residues 1 to 278 shown in Table 1 or a functional fragment thereof.

11. A recombinant organism characterised in that it is transformed with the polynucleotide molecule according to any one of claims 1 to 5.

12. A recombinant organism according to claim 10 selected from the group consisting of entomopoxvirus, nuclear polyhedrosis virus, bacteria, fungi, protozoa and plants.

13. A method for controlling the proliferation of insects, comprising applying to an infested area a recombinant organism according to claim 10 or 11 optionally in admixture with an acceptable agricultural carrier.

AMENDED CLAIMS

[received by the International Bureau on 25 November 1994 (25.11.94); original claims 6 and 7 amended; remaining claims unchanged (1 page)]

1. A polynucleotide molecule comprising a nucleotide sequence which encodes an insecticidal toxin and which is at least 70% homologous to the nucleotide sequence shown in Table 1 from residue 83 to 919, or a fragment thereof which fragment encodes an insecticidal polypeptide.

2. A polynucleotide molecule as claimed in claim 1 in which the nucleotide sequence is at least 90% homologous to the nucleotide sequence shown in Table 1 from residue 83 to 919.

3. A polynucleotide molecule comprising a nucleotide sequence substantially corresponding to the sequence shown in Table 1 from residue 83 to 919 or a fragment thereof, which fragment encodes an insecticidal polypeptide.

4. A polynucleotide molecule according to claim 7, wherein the nucleotide sequence encodes an insecticidal toxin, or an insecticidal fragment thereof, from *Xenorhabdus nematophilus*

5. A polynucleotide nucleotide molecule according to any one of claims 1 to 4 in which the molecule is a DNA molecule.

6. A purified insecticidal protein, or functional fragment thereof, from the bacterial genus *Xenorhabdus*.

7. A purified insecticidal protein, or functional fragment thereof, from *Xenorhabdus nematophilus*.

8. An insecticidal toxin which includes an amino acid sequence which is at least 70% homologous to residues 1 to 278 shown in Table 2 or a functional fragment thereof.

9. An insecticidal toxin as claimed in claim 8 in which the toxin includes an amino acid sequence which is at least 90% homologous to residues 1 to 278 shown in Table 2 or a functional fragment thereof.

10. An insecticidal toxin, the toxin including an amino acid sequence substantially corresponding to residues 1 to 278 shown in Table 1 or a functional fragment thereof.

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl.[6] C12N 15/31, C12N 5/10, C12P 21/02, A01N 63/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Derwent Database: file WPAT: Chemical Abstracts Service: file CASM. See "Electronic database" box for keywords.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: C12N 15/31

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
Derwent database, file WPAT; Chemical Abstracts service, file CASM; Keywords: "Xenorhabdus and (nematophilus or beddingii)"; "Akhurst" (in WPAT), "Akhurst and Xenorhab:" in CASM.
STN International, file CA, sequence "CCGTTGAAAAGCAAA" and "PMEANQQSNA".

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	AU,B,21230/83 (558287) (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL ORGANISATION) 22 May 1984 (22.05.84) See entire specification especially page 3 lines 15-29.	6,7
X	B.V. McINERNEY et al: "Biologically active metabolites from Xenorhabdus spp, Part 1. Dithiolopyrrolone derivatives with antibiotic activity". Journal of Natural Products, Vol. 54, number 3, pp. 774-784, May-June 1991. See abstract, page 779 last 2 paragraphs, page 780 Table 2, page 781 first paragraph	6,7

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
6 September 1994 (06.09.94)	30 SEPTEMBER 1994 (30.09.94)
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. (06) 2853929	Authorized officer R Porter ROBYN PORTER Telephone No. (06) 2832318

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X	B.V. McINERNEY et al: "Biologically active metabolites from Xenorhabdus spp, Part 2. Benzopyran-1-one derivatives with gastroprotective activity", Journal of Natural Products, Vol. 54, number 3, pp. 785-795, May-June 1991.	6,7

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member					
AU 21230/83	CA	1214130	EP	126092	US	4672130
	WO	84/01775	ZA	8307974		

END OF ANNEX

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(57) Abstract

A method for killing pests (e.g. insects) comprising administering material from *Xenorhabdus* species (e.g. *X. nematophilus*) such as cells or supernatants orally to the pests, either alone or in conjunction with *Bacillus thuringiensis* or pesticidal materials derived therefrom. Also disclosed is an isolated pesticidal agent (and compositions comprising the same) characterised in that it is obtainable from cultures of *X. nematophilus* or mutants thereof, has oral pesticidal activity against *Pieris brassicae*, *Pieris rapae* and *Plutella xylostella*, is substantially heat stable to 55 °C, is proteinaceous, acts synergistically with *B. thuringiensis* cells as an oral pesticide and is substantially resistant to proteolysis by trypsin and proteinase K. DNA encoding pesticidal activity is also disclosed.

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PESTICIDAL AGENTS

The present invention relates to materials, agents and compositions having pesticidal activity which derive from bacteria, and more particularly from *Xenorhabdus* species. The invention further relates to organisms and methods employing such compounds and compositions.

There is an ongoing requirement for materials, agents, compositions and organisms having pesticidal activity, for instance for use in crop protection or insect-mediated disease control. Novel materials are required to overcome the problem of resistence to existing pesticides. Ideally such materials are cheap to produce, stable, have a high toxicity (either when used alone or in combination) and are effective when taken orally by the pest target. Thus any invention which provided materials, agents, compositions or organisms in which any of these properties was enhanced would represent a step forward in the art.

Xenorhabdus spp. in nature are frequently symbiotically associated with a nematode host, and it is known that this association may be used to control pest activity. For instance, it is known that certain *Xenorhabdus spp.* alone are capable of killing an insect host when injected into the host's hemocoel.

In addition, one extracellular insecticidal toxin from *Photorhabdus luminescens* has been isolated (this species was recently removed from the genus *Xenorhabdus*, and is closely related to the species therein). This toxin is not effective when ingested, but is highly toxic when injected into certain insect larvae (see Parasites and Pathogens of Insects Vol.2, Eds. Beckage, N. E. et al., Academic Press 1993).

Also known are certain low-molecular weight heterocyclic compounds from *P.luminescens* and *X.nematophilus* which have antibiotic properties when applied intravenously or topically (see Rhodes, S.H. et al., PCT WO 84/01775)..

Unfortunately none of these prior art materials have the ideal pesticide characteristics discussed above, and in particular, they do not have toxic activity when administered orally.

The present invention provides pesticidal agents and compositions from *Xenorhabdus* species, organisms which produce such compounds and compositions, and methods which employ these agents, compositions and organisms, that alleviate some of the problems with the prior art.

According to one aspect of the present invention there is disclosed a method of killing or controlling insect pests comprising administering cells from *Xenorhabdus* species or pesticidal materials derived or obtainable therefrom, orally to the pests.

A PCT application of CSIRO published as WO 95/00647 discloses an apparently toxic protein from *Xenorhabdus nematophilus*; however no details of the protein's toxicity are given, and certainly there is no disclosure of its use as an oral insecticide.

Thus the invention provides an insecticidal composition adapted for oral administration to an insect, which composition comprises a pesticidal material obtainable from a *Xenorhabdus* species, or a pesticidal fragment thereof, or a pesticidal variant or derivative of either of these.

The composition may in fact comprise cells of *Xenorhabdus* or alternatively supernatant taken from cultures of cells of *Xenorhabdus* species. However, the composition

preferably comprises toxins isolable from *Xenorhabdus* as illustrated hereinafter. Toxic activity has been associated with material encoded by the nucleotide sequence of Figure 2. Thus, the composition suitably comprises a pesticidal material which is encoded by all or part of the nucleotide sequence of Figure 2. Pesticidal fragments as well as variants or derivatives of such toxins may also be employed.

The sequence of Figure 2 is of the order of 40kb in length. It is believed that this sequence may encode more than one protein, each of which may regulate or be insecticidal either alone or when presented together. It is a matter of routine to determine which parts are necessary or sufficient for insecticidal activity.

As used herein the term ``variant'' refers to toxins which have modified amino acid sequence but which share similar activity. Certain amino acids may be replaced with different amino acids without altering the nature of the activity in a significant way. The replacement may be by way of ``conservative substitution'' where an amino acid is replaced with an amino acid of broadly similar properties, or there may be some non-conservative substitutions. In general however, the variants will be at least 60% homologous to the native toxin, suitably at least 70% homologous and more preferably at least 90% homologous.

The term ``derivative'' relates to toxins which have been modified for example by chemical or biological methods.

These toxins are novel, and they and the nucleic acids which encode them form a further aspect of the invention.

A preferred *Xenorhabdus* species is the bacteria *X.nematophilus*. Particular strains of *X.nematophilus* which are useful in the context of the invention are

ATTC 19061 strain, available from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (NCIMB). In addition, suitable strains include two novel strains of *Xenorhabdus* which were deposited at the NCIMB on 10 July 1997 and were designated with repository numbers NCIMB 40886 and NCIMB 40887. These latter strains form a further aspect of the invention.

All strains have common characteristics as set out in the following Table 1.

Table 1

Characteristics	Strains ATCC 19061	NCIMB 40887	NCIMB 40886
Gram strain	negative	negative	negative
Shape/size	rods up to 4µm long	rods up to 4µm long	rods up to 4µm long
Motile	Yes	Yes	Yes
Bioluminescent	No	No	No
Colour on NBTA*	blue	blue	blue
insecticidal on ingestion by insects	yes	yes	yes
Production of Antibiotics	yes	yes	yes
Resistant to ampicillin (50µg/ml)	yes	yes	yes
colony morphology/ colour	circular convex cream	circular convex cream	circular convex cream

*NBTA (Oxoid nutrient agar containing 0.0025% bromothymol blue and 0.004% tetrazolium chloride)

Preferably the pest target is an insect, and more preferably it is of the order Lepidoptera, particularly

Pieris brassicae, *Pieris rapae*, or *Plutella xylostella* or the order *Diptera*, particularly *Culex quinquefaciatus*.

In a preferred embodiment of the invention, cells from *Xenorhabdus* species or agents derived therefrom are used in conjunction with *Bacillus thuringiensis* as an oral pesticide.

In further embodiments, rather than using *Bacillus thuringiensis* itself, pesticidal materials obtainable from *B.thuringiensis* (e.g. delta endotoxins or other isolates) are used in conjunction with *Xenorhabdus* species.

The term 'obtainable from' is intended to embrace not only materials which have been isolated directly from the bacterium in question, but also those which have been subsequently cloned into and produced by other organisms.

Thus the unexpected discovery that bacteria of the genus *Xenorhabdus*(and materials derived therefrom) have pesticidal activity when ingested, and that such bacteria and materials can be used advantageously in conjunction with *B.thuringiensis* (and toxins or materials derived therefrom), forms the basis of a further aspect of the present invention. The pesticidal activity of *B.thuringiensis* isolates alone have been well documented. However, synergistic pesticidal activity between such isolates and bacteria of the *Xenorhabdus* species (or materials derived therefrom) has not previously been demonstrated.

In still further embodiments of the invention, culture supernatant taken from cultures of *Xenorhabdus* species, particularly *X. nematophilus*, is used in place of cells from *Xenorhabdus* species in the methods above.

All of these methods can be employed, inter alia, in pest control.

The invention also makes available pesticidal compositions comprising cells from *Xenorhabdus* species, preferably *X.nematophilus*, in combination with *B. thuringiensis*. As with the methods above, a pesticidal toxin from *B.thuringiensis* (preferably a delta endotoxin) may be used as an alternative to *B.thuringiensis* in the compositions of the present invention

Likewise, culture supernatant taken from cultures of *Xenorhabdus* species, preferably, *X.nematophilus* may be used in place of cells from *Xenorhabdus* species.

Such compositions can be employed, *inter alia*, for crop protection eg. by spraying crops, or for livestock protection. In addition, compositions of the invention may be used in vector control.

The invention further encompasses novel pesticidal agents which can be isolated from *Xenorhabdus spp*. Techniques for isolating such agents would be understood by the skilled person.

In particular, such techniques include the separation and identification of toxin proteins either at the protein level or at the DNA level.

The applicants have cloned and partially sequenced a region of DNA from *Xenorhabdus* NCIMB 40887 which region codes for insecticidal activity and this is shown as Figure 2 (SEQ ID NO. 1) hereinafter. Thus in a preferred embodiment the invention also provides a toxin which is encoded by DNA of SEQ ID No. 1 or a variant or fragment thereof.

The invention also provides a recombinant DNA which encodes such a toxin. The recombinant DNA of the invention may comprise the sequence of Figure 2 or a variant or fragment thereof. Other DNA sequences may encode similar proteins as a result of the degeneracy of the genetic code. All such sequences are encompassed by the invention.

The sequence provided herein is sufficient to allow probes to be produced which can be used to identify and subsequently to extract DNA of toxin genes. This DNA may then be cloned into vectors and host cells as is understood in the art.

DNA which comprises or hybridises with the sequence of Figure 2 under stringent conditions forms a further aspect of the invention.

The expression ``hybridises with'' means that the nucleotide sequence will anneal to all or part of the sequence of Figure 2 under stringent hybridisation conditions, for example those illustrated in ``Molecular Cloning'', A Laboratory Manual'' by Sambrook, Fritsch and Maniatis, Cold Spring Habor Laboratory Press, Cold Spring Harbor, N.Y.

The length of the sequence used in any particular analytical technique will depend upon the nature of the technique, the degree of complementarity of the sequence, the nature of the sequence and particularly the GC content of the probe or primer and the particular hybridisation conditions employed. Under high stringency, only sequences which are completely complementary will bind but under low stringency conditions, sequences which are 60% homologous to the target sequence, more suitably 80% homologous, will bind. Both high and low stringency conditions are encompassed by the term ``stringent conditions'' used herein.

Suitable fragments of the DNA of Figure 2, i.e. those which encode pesticidal agents may be identified using standard techniques. For example, transposon mutagenesis techniques may be used, for example as described by H.S. Siefert et al., Proc. Natl. Acad. Sci. USA, (1986) 83, 735-739. Vectors such as the cosmid cHRIMI, can be mutated using a variety of transposons and then screened for loss of insectidal activity. In this way regions of DNA encoding proteins responsible for toxic activity can be identified.

For example, the mini-transposon mTn3(HIS3) can be introduced into a toxic *Xenorhabdus* clone such as cHRIM1, hereinafter referred to as `clone 1', by electroporating cHRIM1 DNA into *E.coli* RDP146(pLB101) and mating this strain with *E.coli* RDP146(pOX38), followed by *E. coli* NS2114Sm. The final strain will contain cHRIM1DNA with a single insertion of the transposon mTn3(HIS3). These colonies can be cultured and tested for insecticidal activity as described in Example 8 hereinafter. Restriction mapping or DNA sequencing can be used to identify the insertion point of mTn3(HIS3) and hence the regions of DNA involved in toxicity. Similar approached can be used with other transposons such as Tn5 and mTn5.

Site directed mutagenesis of cHRIM1 as outlined in ``Molecular Cloning, A Laboratory Manual'' by Maniatis, Fritsch and Sambrook, (1982) Cold Spring Harbor, can also be used to test the importance of specific regions of DNA for toxic activity.

Alternatively, subcloning techniques can be used to identify regions of the cloned DNA which code for insecticidal activity. In this method, specific smaller fragments of the DNA are subcloned and the activity determined. To do this, cosmid DNA can be cut with a suitable restriction enzyme and ligated into a compatible

restriction site on a plasmid vector, such as pUC19. The ligation mix can be transformed into *E. coli* and transformed clones selected using a selection marker such as antibiotic resistance, which is coded for on the plasmid vector. Details of these techniques are described for example in Maniatis et al, supra, (see p390-391) and Methods in Molecular Biology, by L.G. Davies, M.D. Dibner and J.F. Battey, Elsevier, (see p222-224).

Individual colonies containing specific cloned fragments can be cultured and tested for activity as described in Example 8 hereinafter. Subclones with insecticidal activity can be further truncated using the same methodology to further identify regions of the DNA coding for activity.

The invention also discloses an isolated pesticidal agent characterised in that the agent is obtainable from cultures of *X. nematophilus* or variants thereof, has oral pesticidal activity against *Pieris brassicae, Pieris rapae* and *Plutella xylostella*, is substantially heat stable to 55°C, is proteinaceous, acts synergistically with *B.thuringiensis* cells as an oral pesticide and is substantially resistant to proteolysis by trypsin and proteinase K.

By 'substantially heat stable to 55°C' is meant that the agent retains some pesticidal activity when tested after heating the agent in suspension to 55°C for 10 minutes, and preferably retains at least 50% of the untreated activity.

By 'substantially resistant to proteolysis' is meant that the agent retains some pesticidal activity when exposed to proteases at 30°C for 2 hours and preferably retains at least 50% of the untreated activity.

By 'acts synergistically' is meant that the activity of the combination of components is greater than one might expect from the use of the components individually. For example, when used in conjunction with *B.thuringiensis* cells as an oral pesticide, the concentration of B. thuringiensis cellular material necessary to give 50% mortality in a P.brassicae when used alone is reduced by at least 80% when it is used in combination the agent at a concentration sufficient to give 25% mortality when the agent is used alone.

It has been found that the activity of the material is retained by 30 kDa cut-off filters but is only partly retained by 100 kDa filters.

Preferably the agent is still further characterised in that the pesticidal activity is lost through treatment at 25°C with sodium dodecyl sulphate (SDS - 0.1% 60 mins) and acetone (50%, 60 mins).

Clearly the characterising properties of the isolated agent described above can be utilised to purify it from, or enrich its concentration in, *Xenorhabdus* species cells and culture medium supernatants. Methods of purifying proteins from heterogenous mixtures are well known in the art (eg. ammonium sulphate precipitation, proteolysis, ultrafiltration with known molecular weight cut-off filters, ion-exchange chromatography, gel filtration, etc.). The oral pesticidal activity provides a convenient method of assaying the level of agent after each stage, or in each sample of eluent. Such methodology does not require inventive endeavour by those skilled in the art.

The invention further discloses oral pesticidal compositions comprising one or more agents as described above. Such compositions preferably further comprise other pesticidal materials from non-*Xenorhabdus* species.

These other materials may be chosen such as to have complementary properties to the agents described above, or act synergistically with it.

Preferably the oral pesticidal composition comprises one or more pesticidal agents as described above in combination with *B. thuringiensis* (or with a toxin derived therefrom, preferably endotoxin).

Recombinant DNA encoding said proteins also forms a further aspect of the invention. The DNA may be incorporated into an expression vector under the influence of suitable control elements such as promoters, enhancers, signal sequences etc. as is understood in the art. These expression vectors form a further aspect of the invention. They may be used to transform a host organism so as to ensure that the organism produces the toxin.

The invention further makes available a host organism comprising a nucleotide sequence coding for a pesticial agent as described above.

Methods of cloning the sequence for a characterised protein into a host organism are well known in the art. For instance the protein may be purified and sequenced: as activity is not required for sequencing, SDS gel electrophoresis followed by blotting of the gel may be used to purify the protein. The protein sequence can be used to generate a nucleotide probe which can itself be used to identify suitable genomic fragments from a *Xenorhabdus* gene library. These fragments can then be inserted via a suitable vector into a host organism which can express the protein. The use of such general methodology is routine and non-inventive to those skilled in the art. Such techniques may be applied to the production of *Xenorhabdus* toxins other than those encoded by the sequence of Figure 2.

It may be desirable to manipulate (eg. mutate) the agent by altering its gene sequence (and hence protein structure) such as to optimise its physical or toxicological properties.

It may also be desirable for the host to be engineered or selected such that it also expresses other proteinaceous pesticidal materials (eg. delta- endotoxin from *B. thuringiensis*). Equally it may be desirable to generate host organisms which express fusion proteins composed of the active portion of the agent plus these other toxicity enhancing materials.

A host may be selected for the purposes of generating large quantities of pesticidal materials for purification e.g. by using *B.thuringiensis* transformed with the agent-coding gene. Preferably however the host is a plant, which would thereby gain improved pest-resistance. Suitable plant vectors, eg. the Ti plasmid from *Agrobacterium tumefaciens*, are well known in the art. Alternatively the host may be selected such as to be directly pathogenic to pests, eg. an insect baculovirus.

The teaching and scope of the present invention embraces all of these host organisms plus the agents, mutated agents or agent-fusion materials which they express.

Thus the invention makes available methods, compositions, agents and organisms having industrially applicable pesticidal activity, being particularly suited to improved crop protection or insect-mediated disease control.

The methods, compositions and agents of the present invention will now be described, by way of illustration only, through reference to the following non-limiting examples and figures. Other embodiments falling within

the scope of the invention will occur to those skilled in the art in the light of these.

FIGURE

Figure 1 shows the variation with time of the growth of *X. nematophilus* ATCC 19061 and activity of cells and supernatants against *P. brassicae* as described in Example 3.

Figure 2 shows the sequence of a major part of a cloned toxin gene from *Xenorhabdus*.

Figure 3 shows a comparison of the restriction maps of cloned toxin genes from two strains of *Xenorhabdus* (clone 1 above and clone 3 below).

EXAMPLES

Example 1 - Use of *X. nematophilus* cells as an oral insecticide

CELL GROWTH: A subculture of *X.nematophilus* (ATCC 19061, Strain 9965 available from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland) was used to inoculate 250 ml Erlenmeyer flasks each containing 50 ml of Luria Broth containing 10g tryptone, 5g yeast extract and 5g NaCl per litre. Cultures were grown in the flasks at 27°C for 40hrs on a rotary shaker.

PRODUCTION OF CELL SUSPENSION: Cultures were centrifuged at 5000 x g for 10 mins. The supernatants were discarded and the cell pellets washed once and resuspended in an equal volume of phosphate buffered saline (8g NaCl, 1.44g Na_2HPO_4 and 0.24g of KH_2PO_4 per litre) at pH 7.4.

ACTIVITY OF CELL SUSPENSION TO INSECTS: The bioassays were as follows: *P. brassicae*: The larvae were allowed to feed on an artificial agar-based diet (as described by David and Gardiner (1965) London Nature, 207, 882-883) into which a series of dilutions of cell suspension had been incorporated. The bioassays were performed using a series of 5 doses with a minimum of 25 larvae per dose. Untreated and heat-treated (55°C for 10 minutes) cells were tested. Mortality was recorded after 2 and 4 days with the temperature maintained at 25°C.

	LC50 cells/g diet	
Treatment	2 days	4 days
Untreated	5.9×10^5	9.8×10^4
Treated 55°C	7.1×10^5	1.4×10^5

Aedes aegypti: The larva were exposed to a series of 5 different dilutions of cell suspension in deionised water. The biosassays were performed using 2 doses per dilution of 50 ml cell suspension in 9.5cm plastic cups with 25 second instar larvae per dose. Untreated and heat-treated (55°C or 80°C for 10 minutes) cells were tested. Mortality was recorded after 2 days with the temperature maintained at 25°C.

	LC50 cells/ml
Treatment	2 days
Untreated	5.1×10^6
Treated 55°C	7.4×10^6
Treated 80°C	$> 10^8$

Culex quinquefaciatus: The larvae were exposed to a single concentration cell suspension containing 4×10^7 cells/ml. The biosassays were performed using 2 50 ml cell suspensions in 9.5 cm plastic cups with 25 second instar larvae per cup. Untreated and heat-treated (55°C or 80°C for 10 minutes) cells were tested. Mortality was

recorded after 2 days with the temperature maintained at 25°C.

Treatment	% Mortality 2 days
Untreated	100
Treated 55°C	100
Treated 80°C	0

Thus these results clearly show that cells from *X. nematophilus* are effective as an oral insecticide against a number of insect species (and are particularly potent against *P.brassicae*). The insecticidal activity is not dependent on cell viability (i.e is largely unaffected by heating to 55°C which reduces cell viability by >99.99%) but is much reduced by heating to 80°C, which denatures most proteins.

Example 2 - Use of *X.nematophilus* supernatant as an oral insecticide

CELL GROWTH: Cultures were grown as in Example 1.

PRODUCTION OF SUPERNATANT: Cultures were centrifuged twice at 10000g for 10 mins. The cell pellets were discarded.

ACTIVITY OF SUPERNATANT TO INSECTS: The Bioassay was as follows:
Activity against neonate *P. brassicae* and two day old *Pieris rapae* and *Plutella xylostella* larvae was measured as for *P. brassicae* in Example 1, but using a series of untreated dilutions of supernatant in place of of cell supensions and with mortality being recorded after 4 days only.

Insect species	LC50 (µl supernatant/g diet) 4 days
P. brassicae	22
P. rapae	79
P. xylostella	135

In addition, size-reducing activity (62% reduction in 7 days) against *Mamestra brassicae* was detected in larvae fed on an artificial diet containing *X. nematophilus* supernatant (results not shown).

Thus these results clearly show that the supernatant from *X. nematophilus* culture medium is effective as an oral insecticide against a number of insect species, and are particularly potent against *P. brassicae*.

The heating of supernatants to 55°C for 10 minutes caused a partial loss of activity while 80°C caused complete loss of activity. Activity was also completely lost by treatment with SDS (0.1%w/v for 60 mins) and Acetone (50% v/v for 60 mins) but was unaffected by Triton X-100 (0.1% 60 mins), non-diet P40 (0.1% 60 mins), NaCl (1 M for 60 mins) or cold storage at 4°C or -20°C for 2 weeks. All of these properties are consistent with a proteinaceous agent.

The general mode of action of *X. nematophilus* cells and supernatants i.e. reduction in larval size and death within 2 days at high dosages, and other properties, eg. temperature resistence, appear to be similar suggesting a single agent or type of agent may be responsible for the oral insecticide activity activities of both cells and supernatants.

Example 3 - Timescale for appearance of ingestable insecticidal activity

CELL GROWTH: 1ml of an overnight culture of *X. nematophilus* was used to inoculate an Erlenmeyer flask. Cells were then cultured as in Example 1. Growth was estimated by measuring the optical density at 600 nm.

PRODUCTION OF CELL SUSPENSION AND SUPERNATANTS: These were produced as in Examples 1 and 2.

ACTIVITY OF CELLS AND SUPERNATANTS AGAINST *P. BRASSICAE*: The cell suspension bioassay was carried out as in Example 1, but using a single dose of suspended cells equivalent to 50 µl of broth/g diet and measuring mortality after 2 days. The cell supernatant bioassay was carried out as in Example 2, but using a single dose equivalent to 50 µl supernatant/g diet (i.e. more than twice the LC50) and measuring mortality after 2 days.

The results are shown in Fig. 1. Thus these results clearly show that cells taken from *X. nematophilus* culture medium are highly effective as an oral insecticide against *P. brassicae* after only 5 hours, and supernatants are highly effective after 20 hours. Although some slight cell lysis was observed in the early stages of growth, no significant cell lysis was observed after this point demonstrating that the supernatant activity may be due to an authentic extracellular agent (as opposed to one released only after cell breakdown).

Example 4 - Synergy between *X. nematophilus* cells and *B.thuringiensis* powder preparations

CELL GROWTH AND SUSPENSION: *X. nematophilus* cells were grown and suspended as in Example 1. *B. thuringiensis* strain HD1 (from *Bacillus* Genetic Stock Centre, The Ohio State University, Columbus, Ohio 43210, USA) was cultured, harvested and formulated into a powder as described by Dulmage *et al.*(1970) J. Invertebrate Pathology 15, 15-20.

ACTIVITY OF *X. NEMATOPHILUS* CELLS AND *B. THURINGIENSIS* POWDER AGAINST *P. BRASSICAE*: The bioassays was carried out using *X. nematophilus* and *B. thuringiensis* in combination or using *B. thuringiensis* cell powder alone. Bioassays were carried out as in Example 1 but with various dilutions of *B. thuringiensis* powder in place of *X. nematophilus*. For the combination experiment, a constant dose of *X. nematophilus* cell suspension sufficient to give 25% mortaility was also added to the diet. Mortality was recorded after 2 days.

	LC50 (µg Bt powder/g diet)
Bioassay	2 days
B.t. alone	1.7
B.t. plus *X.nematophilus*	0.09

These results clearly demonstrate the synergism between *X. nematophilus* cells and *B. thuringiensis* powder when acting as an oral insecticide against *P. brassicae*.

Example 5 - Synergy between of *X.nematophilus* supernatants and *B. thuringiensis* powder

CELL GROWTH AND PRODUCTION OF SUPERNATANTS: *X. nematophilus* cells were grown and supernatants prepared as in Example 2. *B. thuringiensis* was grown and treated as in Example 4.

ACTIVITY OF *X. NEMATOPHILUS* SUPERNATANTS AND Bt CELL POWDER AGAINST *P. BRASSICAE*:
The bioassays were carried out using *X. nematophilus* supernatants and *B. thuringiensis* in combination or using *B. thuringiensis* powder alone. The Bioassay against neonate *P. brassicae* and two day old *Pieris rapae* and *Plutella xylostella* larvae were measured as in Example 2 but with various dilutions of *B. thuringiensis* in place of *X. nematophilus*. For the combination experiment, a

constant dose of *X. nematophilus* supernatant sufficient to give 25% mortality was also added to the diet. Mortality was recorded after 4 days.

LC_{50} (μg Bt powder/g) diet

Insect species	Bt alone	Bt plus Xn
P. brassicae	1.4	0.12
P. rapae	2.5	0.26
P. xylostella	7.2	0.63

These results clearly demonstrate the synergism between *X.nematophilus* supernatants and *B.thuringiensis* powder when acting as an oral insecticide against several insect species. The fact that both *X. nematophilus* cells and supernatants demonstrate this synergism strongly suggests that a single agent or type of agent is responsible for the demonstrated activities.

Example 5 - Characterisation of insecticidal agent from *X.nematophilus* supernatant by proteolysis

CELL GROWTH AND PRODUCTION OF SUPERNATANTS: *X. nematophilus* cells were grown and supernatants prepared as in Example 2.

PROTEOLYSIS OF SUPERNATANT: Culture supernatant (50ml) was dialysed against 0.5 M NaCl (3 x 1 l) for 48 hours at 4°C. The volume of the supernatant in the dialysis tube was reduced five-fold by covering with polyethylene glycol 8000 (Sigma chemicals). Samples were removed and treated with either trypsin (Sigma T8253 = 10,000 units/mg) or proteinase K (Sigma P0390 = 10 units/mg) at a concentration of 0.1 mg protease/ml sample for 2 hours at 30°C.

ACTIVITY OF PROTEASE TREATED SUPERNATANT AGAINST *P. BRASSICAE*: The boassay against neonate *P. brassicae*

larvae was carried out by spreading 25 µl of each 'treatment' on the artificial agar-based diet referred to in Example 1 in a 4.5 cm diameter plastic pot. Four pots each containing 10 larvae were used for each treatment. Mortalities were recorded after 1 and 2 days. Controls using water only, trypsin (0.1 mg/ml) and proteinase K (0.1 mg/ml) were also tested in the same way.

	% Mortality	
Treatment	1 day	2 days
Untreated supernatant	60	100
Proteinase K treated supernatant	45	100
Trypsin treated supernatant	40	100
All controls (no supernatant)	0	0

Example 6

Entomocidal activity of other *Xenorhabdus*

Using the methodology of Examples 1 and 2, four different *xenorhabdus* strains were tested against insect pests. The results obtained were as follows:

I) Activity to *Pieris brassicae*

Strain deposit no/code	Cells 10^6/grm diet % mortality	Supernatant LC50 µl/gram of diet
NCIMB 40887	100	0.09
0014	100	0.52
0015	80	3.73
NCIMB 40886	100	0.05

It was found that entomocidal activity of cells and supernatant was reduced by more than 99% when all four strains were heated at 80°C for 10 minutes.

II) Activity to mosquitoes (*Aedes aegypti*)
Bacteria added at the rate of 10^7cells/ml of water

Strain deposit no/code	Cells 10^6/grm diet % mortality
NCIMB 40887	0
0014	40
0015	45
NCIMB 40886	95

Furthermore, all strains significantly reduced the growth of *Heliothis virescens*.

Example 7
Cloning of toxin genes from strains of *Xenorhabdus*

Total cellular DNA was isolated from NCIMB 40887 and ATCC 19061 using a Quiagen genomic purification DNA kit. Cells were grown in L borth (10g tryptone, 5g yeast extract and 5g NaCl per 1) at 28°C with shaking (150rpm) to an optical density of 1.5 A_{600}. Cultures were harvested by centrifugation at 4000xg and resuspended in 3.5mls of buffer B1 (50mM Tris/HCl, 0.05% Tween 20, 0.5% Triton X-100, pH7.0) and incubated for 30 mins at 50°C. DNA was isolated from bacterial lysates using Quiagen 100/G tips as per manufacturers instructions. The resulting purified DNA was stored at -20°C in TE buffer (10mM Tris, 1mM EDTA, pH 8.0).

A representative DNA library was produced using total DNA of NCIMB 40887 and ATTC 19061 partially digested with the restriction enzyme *Sau3a*. Approximately 20µg of DNA from each strain was incubated at 37°C with 0.25 units of the enzyme. At time intervals of 10, 20, 30, 45 and 60 minutes, samples were withdrawn and heated at 65°C for 15 minutes. To visualise the size of the DNA fragments, the samples were electrophoresed on 0.5% w/v agarose gels.

The DNA samples which contained the highest proportion of 30 to 50kb fragments were combined and treated with 4 units of shrimp alkaline phosphatase (Boehringer) for 15 minutes at 37°C, followed by heat treatment at 65°C to inactivate the phosphatase.

The size selected DNA fragments were ligated into the BamH1 site of the cosmid vector SuperCos! (Stratagent) and packaged into the *Escherichia coli* strain XL Blue 1, using a Gigapack II packaging kit (Stratgene) in accordance with the manufacturers instructions.

To select for cosmid clones with entomocidal activity, individual colonies selected on L agar plates containing 25µg/ml ampicillin, were grown in L broth (containing 25µg/ml ampicillin) overnight at 28°C. Broth cultures (50µl) were individually spread onto the surface of insect diet contained in 4.5cm diameter pots, as described in Example 5. To each container 10 neonate *P. brassicae* larvae were added. Larvae were examined after 24, 72 and 96 hours recording mortality and size of surviving larvae. A total of 220 clones of NCIMB 40887 were tested, of which two were found to cause reduction in larval growth and death within 72 hours. Of 370 clones from ATTC 19061, one was found to cause larval death within 72 hours.

Example 8

Activity of cloned toxin genes to *Pieris brassicae*

The three active clones from Example 7 were grown in L broth, containing 25µg/ml ampicillin, for 24 hours at 28°C, on a rotary shaker at 150rpm. The activity of the toxin clones to neonate larvae were performed by incorporation of whole broth cultures into insect diet, as described in Example 1.

Clone No	Strain	LC50 (µl broth/g insect diet)
1	NCIMB 40887	13.03
2	NCIMB 40887	16.7
3	ATTC 19061	108.7
Control*		No effect at 100µl/g

*XL1 Blue *E. coli* broth

When *E. coli* toxin clones were heated at 80°C for 10 minutes and added to the diet at a rate of 100µl/g, no activity to larvae was detected. Highlighting the heat sensitivity of the toxins.

Example 9

Sequencing of the cloned toxin from NCIMB 40887

Cosmid DNA of the entomocidal clone 1 above from NCIMB 40887 was purified using the Wizard Plus SV DNA system (Promega) in accordance with the manufacturers instructions. A partial map of the cloned fragment was obtained using a range of restriction enzymes *Eco*R1, *Bam*H1, *Hind*III, *Sal*1 and *Sac*1 as shown in Figure 3. DNA sequencing was intiatiated from pUC18 and pUC19 based sub-clones of the cosmid, using the enzymes *Eco*R1, *Bam*H1, *Hind*III, *Eco*RV and *Pvu*II. Sequence gaps were filled using a primer walking approach on purified cosmid DNA. Sequence reactions were performed using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmmpliTaq DNA polymerase FS according to the manufacturers instructions. The samples were analysed on an ABI automated sequencer according to the manufacturers instructions. The major part of the DNA sequence for the cloned toxin fragment is shown in Figure 2.

Example 10

Restriction map of cloned toxin from clone 3

Cosmid DNA of the entomocidal clone 3 above was purified as described in Example 9. A restriction map of the cloned fragment was obtained using the restriction enzymes *Bam*H1, *Hind*III, *Sal*1 and *Sac*1 and this is shown in Figure 3. When compared with the map from clone 1 (Figure 3) it is clear that over the regions which overlap, the restriction maps are very similar. The only detectable difference between the two clones was a reduction in size of two *Hind*III fragments in clone 3, corresponding to the 11.4kb and 7.2kb *Hind*III fragments in clone 1 by approximately 2Kb and 200bp respectively. These results indicate the overall relatedness of the DNA region coding for toxicity in the two bacterial strains.

Example 11

Southern Blot Hybridisation Experiments

A 10.3kb *Bam*H1-*Sal*1 fragment of the DNA from clone 1 was used as a probe to hybidise to total *Hind*III digested DNA of the *Xenorhabdus* strains ATCC 19061, NCIMB 40886 and NCIMB 40887. Hybridisation was performed with 20ng/ml of DIG labelled DNA probe at 65°C for 18 hours. Filters were washed prior to immunological detection twice for 5 minutes with 2 x SSC (0.3M NaCl, 30mM sodium citrate, pH 7.0)/0.1% (w/v) sodium dodecyl sulphate at room temperature, and twice for 15 minutes with 0.1 x SSC (15mM NaClm 1.5 mM sodium citrate, pH 7.0) plus 0.1% sodium dodecyl sulphate at 65°C. The probe was labelled and experiments performed in accordance with manufacturers instructions, using a non-radioactive DIG DNA labelling and detection kit (Boehringer). The probe hybridised to a *Hind*III fragment of approximately 8kb in all three strains as well as an 11.4kb fragment in NCIMB 40887 and an approximate 9kb fragment in both NCIMB 40886 and ATCC 19061. These results show that strains NCIMB

40886 and ATCC 19061 contain DNA with close homology to the toxin gene of clone 1 above, confirming the similarity between the toxins produced by the three strains.

CLAIMS

1. An insecticidal composition adapted for oral administration to an insect comprising a pesticidal material obtainable from a *Xenorhabdus* species, or a pesticidal fragment thereof, or a pesticidal variant or derivative of either of these.

2. A composition according to claim 1 wherein the said pesticidal material comprises material encoded by the nucleotide sequence of Figure 2 or variant or fragment thereof, or a sequence which hybridises with said sequence.

3. A composition according to claim 1 or claim 2 which comprises cells of *Xenorhabdus*.

4. A composition as claimed in any one of the preceding claims which comprises supernatant taken from cultures of cells of *Xenorhabdus* species.

5. A composition according to any one of the preceding claims wherein the *Xenorhabdus* species is *Xenorhabdus nematophilus*.

6. A composition according to any one of claims 1 to 4 wherein the *Xenorhabdus* species is ATCC 19061, NCIMB 40886 or NCIMB 40887.

7. A composition as claimed in any one of the preceding claims which comprises a further pesticidal material not obtainable from *Xenorhabdus*.

8. A composition according to claim 7 wherein the said further pesticidal material comprises a material obtainable from *B. thuringiensis*.

9. A composition according to claim 8 which further comprises cells of *B. thuringiensis*.

10. A composition according to claim 8 wherein the pesticidal materials obtainable from *B.thuringiensis* comprises the delta endotoxin.

11. A composition according to any one of the preceding claims which further comprises an agriculturally acceptable carrier.

12. A composition according to claim 10 wherein the carrier comprises items of insect diet.

13. A method for killing or controlling insect pests, which method comprises administering to a pest or the environment thereof a composition according to any one of the preceding claims.

14. A method as claimed in claim 12 wherein the pests are insects from the order Lepidoptera or Diptera.

15. A microorganism comprising *Xenorhabdus* strain NCIMB 40886.

16. A microorganism comprising *Xenorhabdus* strain NCIMB 40887.

17. A pesticidal agent which comprises a a toxin comprising a protein which is encoded by DNA which includes SEQ ID No. 1 or a variant or fragment thereof.

18. An isolated pesticidal agent characterised in that it is obtainable from cultures of *X. nematophilus* or mutants thereof, has oral pesticidal activity against *Pieris brassicae*, *Pieris rapae* and *Plutella xylostella*, is substantially heat stable to 55°C, is proteinaceous, acts synergistically with *B. thuringiensis* cells as an

oral pesticide, and is substantially resistant to proteolysis by trypsin and proteinase K.

19. An isolated pesticidal agent as claimed in claim 18 further characterised in that the pesticidal activity is substantially destroyed by treatment with sodium dodecyl sulphate or acetone or heating to 80°C.

20. An isolated pesticidal agent as claimed in claim 18 or claim 19 further characterised in that the agent is an extracellular protein.

21. A recombinant DNA which encodes a pesticidal agent according to any one of claims 17 to 20.

22. A recombinant DNA of claim 21 which comprises the sequence of Figure 2 or a variant or fragment thereof.

23. A recombinant DNA which comprises or hybridises under stringent conditions with all or part of the sequence of Figure 2, and which encodes a pesticidal material.

24. An expression vector comprising a recombinant DNA according to any one of claims 21 to 23.

25. A host organism which has been transformed with an expression vector according to claim 24.

26. A host organism as claimed in claim 25 which has been engineered or selected such that it also expresses other pesticidal proteinaceous toxicity enhancing materials

27. A host organism comprising a nucleotide sequence coding for a fusion protein comprising a pesticidally active portion of an agent as claimed in any one of claims 17 to 20 in combination with other pesticidal proteinaceous toxicity enhancing materials.

28. A host organism as claimed in claim 27 wherein the pesticidal toxicity enhancing materials comprise delta-endotoxin from B. thuringiensis.

29. A host organism as claimed in any one of claims 25 to 289 wherein the host is a plant.

30. A host organism as claimed in any one of claims 25 to 28 wherein the host is a virus pathogenic to insects.

31. A fusion protein as expressed by a host as claimed in claim 27.

32. An pesticidal composition comprising one or more agents as claimed in any one of claims 17 to 20.

Fig.1.

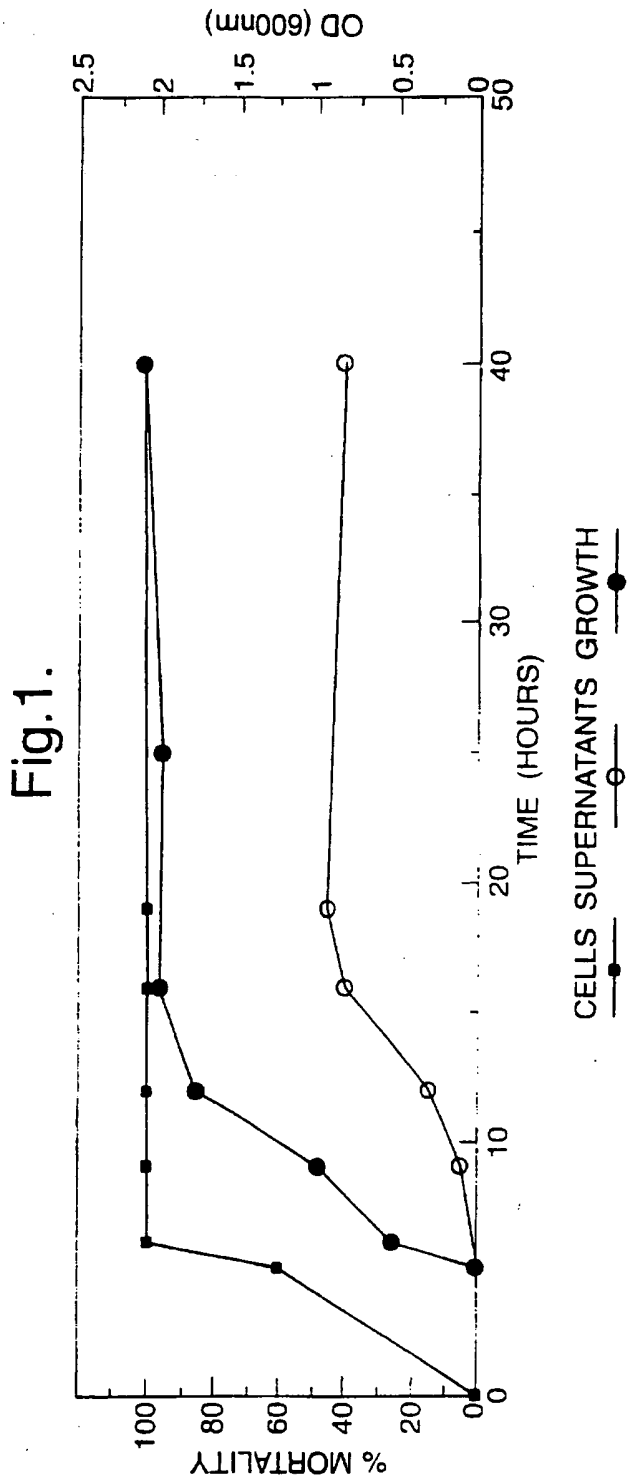
OD (600nm)
2.5
2
1.5
1
0.5
0
50
40
30
20
10
0
TIME (HOURS)
CELLS SUPERNATANTS GROWTH
% MORTALITY
100
80
60
40
20
0

Fig.2.

```
   1  TCCACAATTG CCGGAGAAAA TCAGTCGGGA ACTGCCGGTG ATTATTCGTC ACTTATTAAA
  61  CGAATTTGCC GACCAGAATA AGGCTAAAAA ACTGCTACAG GCGCAACGCG ACTCGAACGA
 121  AGCGTTAACG GTAAAGAGTC ATTCGGATCC GCTGTATCGC TTTTGTGGTT ATCTGGTGTC
 181  TGTCAATGAT ATGACCGGAA TGAAGATGGG CAATAAAAAC ATTAGCCCAC GAGCACCGAG
 241  ATTGTACTTG TATCATGCCT ATCTCTCTTT TATGGAAGCG CACGGCTTTG AACGTCCGTT
 301  AACACTGACT AAGTTTGGTG AATCCATCCC CAAGATTATG CTGGAATACC GGAAGGAGTA
 361  TCGAAAAGTG CGAACCAAGA AAGGCTATTC CTATAACGTG GAATTATCGG AAGAGGCCGA
 421  AGAATGGCTA CCGTCAGTGC CTGAGTGTCG AGACTTTAAA TCACCTGTAT AAAACTTTGA
 481  GCTTTAAGTC TGCACTCCAT ACACAACTTA AAATATCTAA TTGTATTTAA AAGAAAATAA
 541  TAGATGTATA GTTATTTTTT AACTATACAT AAGCTCTACA TGCTCTTCAT TCGTGTAAAA
 601  AATGGGTGAA CAGGTGATAC AGTCAGTGAA TATCATATTA ATTACCGTAA ACCCAGATGT
 661  AGCAAGGCTT TCAGGGAATT GTGCAGAGGG TGCATAACTG AGAGGGTGAA AAAGATTTTC
 721  AGGGGGGCTT ATGGCAGGTA AACAAAATCA GAAGCAAATA CCGTGCACAA TCTGGTTTTT
 781  ATTTTTTGGT ACTACCTCAA ATTAAAATGA TGTAATCATC TGATTTTATT TAAGAATAGA
 841  AGTTAATCAC AATTTCATTG ATGGACTTTC ATTCACACTG GTATAGATAA ATAATTCTGT
 901  TATATCCTGT TTCATTACGC ATTCATCAGG AGTGCTGTTA CAGGAGACAA GAATGTCACA
 961  CATCATTTAC TTGTCGTTAA AGGGCAAGAA GCAGGGTTTA ATTTCAGCGG GTTGTTCAAC
1021  GCCTGAATCA ATTGGAAATC GCTATCAAAA AGGACGTGAA GATCAAATAC AGGTATTGAG
1081  CCTGAATCAT TCGATGAGCC GTGACCAGAA TGTTAATCAT CAACCCGTCA GTTTTGTGAA
1141  ACCCATTGAT AAATCCTCTC CCCTGTTTGC TGGATGCCAG TTTTGTGCAT TACAGGACAA
1201  GCCAGATGGG ACAACTGGAG TTCTTTTATG AAATCAAGCT GACCAGTGCC ACGATTGTGG
1261  ATATTTCCTA TAATTATCCG GCATTCAATC AATGATAATG GTGCGATACC CCATGAAGTG
1321  GTGATGCTCG ATTATAAGTC CATTTCATGC AACCACATCG CCGCAGGACT TCGGGCTACA
1381  GCATACGCAA TTAGCCGGAA GTGAAGAAGC AAGCCGCTTT TATCTGGGGT CTCGAATGTT
1441  AAGCCACTTA AGAAGCCGCT GGTTGAAGAA ACCCCGGTAA AACCCGCTAA ACATCATGCC
1501  CGTTATCGTT GTGTGGATGA TGACGGCAAT CTTTTAACCG AACGCAAGTA TCGGGTTTGC
1561  CTGCCGGATG GTCAGATAAA AGAAGGAAAG ACTGATAAAC AAGGTTACAC CCAATGGCAT
1621  CTTACGGATG ACAAAAATAA ACTTGAATTT CATATTTTAA AGGATTAATA CCATGCCAGC
1681  CTATACCGTT CAGACAAAAA TAGAATCCAA CGTACCTGTT GAAAACCTGC TTTACGACTT
1741  AACCATTTAT CGTAAGGATG CAAAAGGAAA TTTCCATATC TTGCTTGATG TTTTTCAGGA
1801  GAAACTACAG AGTAATTATG AAACACAACA GCATATCACG CAGGAAATAG ACGACGATCT
1861  TTCTGTGATT TATATTATGC AAATTATGCT TCACCGCAAA CATGGCTCAA ATATATTTCC
1921  GGCACTGCAA ACCCATTTTA AGAAAATGTA TACCCTCGGT GAATTAACTT CCGGTAAAGC
1981  CTGTTCGGAG AAAAAACGGG AAAATGCCTG TTATTTTGAA AGTACAGTTG AAACAAAACC
2041  TGTCAGCGAC GGGGATAATA CCGTTGACTT AAATATCACT ATTCCTGAAC GACCTTTTAT
2101  TGCCAAAGAA TATCCCATTG GTCACCCACA CGATCCATTT GAAAAAAGTA AAATTGAATC
2161  ATAAATACAG GACAGGTTAT CGAAAAGAAT TTATCCGGAT CAAAATGGAG CAAGTTTATG
2221  TCAGGGCGCG AGCACACTAT TTTAGCTGCG TTTTTAAGAT GATTATCTCT TAATGTTCAG
2281  TTTTAATAGT GTTTTTATCG AGTGAAATTT AATCGCACAG GCAATTCTTT AGACTTTTAT
2341  AGAAAACTAA AGAATTAAAG AACAAGATTG ACATTTTAAG TTCAAATATT AATCAAAGTA
2401  TGCTCGCGCC CTGAGTTTAT GTGGCCCTGC CGCTTTTTTT TATTGCCTGC CAATAGATAG
2461  ACCAGATATT TATGAGCAAG CGGCACGAGA ATTATGGCAA TATGGCCGAA CTAAAATTGG
2521  TCAACTGGAA ATTAAGCCGG GTGAGGGTTG CCGACATCCT AAAGGTACTT TTTATAATCA
2581  ATATGGTGAA AGAATATCTG GGTTAGATTG GCTGACATTG GCAAGCCTAA GAGATTCAGA
2641  AAATATGATG ATGAGGTTGA TGATGAAGTA GCTGGTATTA CAATGTGGGG AAAATTGACA
2701  GAATGGTTTG AAAAATCAGG GTATGAAAAA GTATTTAGTA ATGTCGGCTT ATCCCATTCT
2761  AATATAAATG ACATAGTAAC TCTTAGTGAT TACTATAACA AAGGATATCA TGTTGTTACT
2821  TTGATTTCAG CAGGAATGTT ATCAGATTTT GGTGACATAG AAACATCAGG AAAAAATCAT
2881  TGGATAGTTT GGGAAGGAGT AGTAGAAAAC TATGAGAAAG AAAATATCAC AAATAATTCA
2941  GATCTGAATC AATATGTAAA TTTAAATCTG TTTTCATGGG GTAAAGTGGA ACATCAAATT
3001  AAAAAAAACA AATCACTAGA TTATGTACTC AACCATATTT TTTGAGGGTT GGTTTTTAAA
3061  CCAATGAAAT AACATGAAAA AAATATTAAT TATTTTTATT TTTTTACTTT ATGGTTGTGG
3121  TAATCCAACG CCAAAAGTTT TACCAAAATC AGAGTTTCTT CCTGATGCAG TGATAAATGA
3181  ACCATATCAG GCATCAATTA CCATCACAGG AGGTGCATTG AATGAAAAAA GCGTTTGGGT
3241  AAAAATTCAT CCTACTGGCT CAGGACTAAC ATGGAATCCA AAAGATAGTT CTTTCCTATA
3301  GGGTGGAAAA AAAGAAATAA GAAAAGATTA TCATCATATA AATATAACAG GTACCCCAAA
3361  GAAGACAGAA TTGATAAAAA TTGAAGTGGT AGGATTTACA TTGGGTACAA TGTACGCACG
3421  GAAAGAGTTC ACTATAAATT ATACTATAAA AGTAAGGGAA TAATTGTCAC TATCAGAATG
3481  GTGATTTAAT TCGCCATTTT TATACTTTTG TATACTCTCT CAACATAATC AGGATTCTTT
```

Fig.2.

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3541  CTTATTATTT TTCATGGTGC TAAAAACGTT TATTGCAAAA ATAAATTAAG TTAATCAGAT
3601  AAATTATCTG CATTACTGTT ATAATCGATA ACACGATAAC CTGACTTTCT GCCTGTTCTT
3661  ATGAACTCGA AGATAATCCT TTCTGAGCCT GAACGAATCA CATTGCAACC ACTCGCTTTG
3721  AATCACCCAC ACCGGGACAT TCGTACGCGA GGAACGGGTT TACTCATGCT TGCCAGAGGG
3781  AGCAAGCCGT CCCAGATCAC CGCTGAAATC GGATGCAGTC TCCGGGTTAT CTGTAATTGG
3841  GTTCACATGT GGCACAGATA GCGGGATTAT TCGGCGGTCA TGCCGGAGGC CGGTATCTCG
3901  CCATGACGCC TGACATGATT GCCACTGCGC TCGAAGCCGC CAGCGCAGAG TCCCTGACGT
3961  GCGTCGAAGC CAGGCAGGGT TTCCCTGCCT TGTACGCTTG AAACGCTGGC GAATACCCTG
4021  AAAAAACAGG GGCTCCCCTA TAAACGCCCC CGCCTGTCGC TTAAAAAAAG CGCAATAAAA
4081  CGGAGTTTGC TGAAAAATCC GCCTTGCTGA ATAAAATTAA GGCCGGAGCA CAGTCAGGAC
4141  ATTACCGTCT GGTCTATTTT GAGTTCTGGG GGCGTTAAAT TACACGGATA ACACGCTGTT
4201  TTACCAGACA ACGTCAGGCA GTATCACGCG AGATGACGTG ATTGATTTTT TAGAGCCGGT
4261  GGCCAGACAA GGGACAACCG CCTGACATTT TTAGTGTTGG ATAATGCGCG TATCCATCAC
4321  GGGATAGAGG AAAAAATCAG AAATGGCGGG TGACGAGAAC ACAACCTGTT TTTATTCTAT
4381  CTTCCCGCTT ACAGCCCAGA GCTGTATCTG ATTGAAATCG TCTGGAAACA GGCCAAATAC
4441  GACTGGCGAC GTTTTATCAC CTGGACTCAG GATACAATGG AATATGAGGT AAATACTTTA
4501  TTGAAAGGTT ATGGCGACCA ATTTGCAATT AACTTTTCTT GAGTACTTAG TAAGAATAGA
4561  GTCAGTCGAG GTTTTTTCAT TTCGGGTCGT GGGGATGATA CTGAAAATTT GTTTGTAATC
4621  TCTGAAAATT GCTGTTTCTG TGGCTACGTC TGTCTTTTGG GATATTGTTT CCATCAAGTC
4681  TGTCAACATA CTGTTAAGTT AGATGTTGAT AAAAGAGACT GAATTATAAT ACAAAACAAT
4741  AAATCACTTG GACAATATTT TATTTCACAT GAGACATTAA GGTTGATTTT CCCAATCTGG
4801  TCAGTTATAA CCGAATAAGG ATCTTGAAAA ATCATGGGAT CTTACTTTTA TCAAATGAAG
4861  TTAACGTAAA AGTTGATAAA GAAAATTATT TAATTCTAAG TGCCGTTGGC ATAAATATTT
4921  TGTGTTTTGT TAATGAATGA ATAACCAGGT AAGCTGGATT TTCATTTTTT AATTACTCGT
4981  TACAATATGC TATTTATTTA TATAAAGAGT TTGTGCCCAT TTAACCAGTA AACAAATTTG
5041  TTCAACCGTA ACTTAGCTTC ATCGACTTTT GGCCTCGCCT GGTCAGAATC TAGGGCCGTT
5101  ATCCTATTTA TTTATGATAA ATAAAATTTA ATTATCTTTA ATAAGCTGAA TATGTGGATT
5161  TGTGCTCAAT CTTGGATTCA AGTATGTATT CCTTTTGGTA CCCTGCTTTA TTTTAAGGCA
5221  GATGAAGAGG ATGCCAACAT GACACAATAT CGATTACGAC TGTAACATTA AAGTCAGTTA
5281  TAAATTTTAT GATTAAAATG AAATTTTAGT AGAAAATCGT ATTCTATTCC GCCATTTACA
5341  ATAGCATCCT CTTTAATATC ATTAATCTCA GATAAAACAA ATAATTACAA TGTGAATAGA
5401  ATAATGACTT ACAAAATAAG CACTAAATCT TCAGATGAAC TCTTAACTGA CAACACTATT
5461  TTATAAAATA ATTGAGGTTA TTATGTATAG CACGGCTGTA TTACTCAATA AAATCAGTCC
5521  CACTCGCGAC GGTCAGACGA TGACTCTTGC GGATCTGCAA TATTTATCCT TCAGTGAACT
5581  GAGAAAAATC TTTGATGACC AGCTCAGTTG GGGAGAGGCT CGCCATCTCT ATCATGAAAC
5641  TATAGAGCAG AAAAAAAATA ATCGCTTGCT GGAAGCGCGT ATTTTTACCC GTGCCAACCC
5701  ACAATTATCC GGTGCTATCC GACTCGGTAT TGAACGAGAC AGCGTTTCAC GCAGTTATGA
5761  TGAAATGTTT GGTGCCCGTT CTTCTTCCTT TGTGAAACCG GGTTCAGTGG CTTCCATGTT
5821  TTCACCGGCT GGCTATCTCA CCGAATTGTA TCGTGAAGCG AAGGACTTAC ATTTTTCAAG
5881  CTCTGCTTAT CATCTTGATA ATCGCCGTCC GGATCTGGCT GATCTGACTC TGAGCCAGAG
5941  TAATATGGAT ACAGAAATTT CCACCCTGAC ACTGTCTAAC GAACTGTTGC TGGAGCTATT
6001  ACCCGCAAGA CCGGAGGTGA TTCGGACGCA TTGATGGAGA GCCTGTCAAC TTACCGTCAG
6061  GCCATTGATA CCCCTTACCA TCAGCCTTAC GAGACTATCC GTCAGGTCAT TATGACCCAT
6121  GACAGTACAC TGTCAGCGCT GTCCCGTAAT CCTGAGGTGA TGGGGCAGGC GGAAGGGGCT
6181  TCATTACTGG CGATTCTGGC CAATATTTCT CCAGAACTGT ATAACATTTT GACCGAAGAG
6241  ATTACGGAAA AGAACGCTGA TGCTTTATTT GCGCAAAACT TCAGTGAAAA TATCACGCCC
6301  GAAAATTTCG CGTCACAATC ATGGATAGCC AAGTATTATG GTCTTGAACT TTCTGAGGTG
6361  CAAAAATACC TCGGGATGTT GCAGAATGGC TATTCTGACA GCACCTCTGC TTATGTGGAT
6421  AATATCTCAA CGGGTTTAGT GGTCAATAAT GAAAGTAAAC TCGAAGCTTA CAAAATAACA
6481  CGTGTAAAAA CAGATGATTA TGATAAACAT GTAAATTACT TTGATCTGAT GTATGAAGGA
6541  AATAATCAAT TCTTTATATG TGCTAATTTT AAGATATCGA GAGAATTTGG GGCGACTCTT
6601  AGGAAAAACT CAGGGACAAG TGGCATTGTC GGCAGCCTTT CCGGTCCCCT GGTAGCCAAT
6661  ACTAATTTCA AAAGCAATTA CTTAAGTAAC ATATCTGATA ATGAATACAG AAATGGCGTA
6721  AAAATATATG CCTATCGCTA TACGTCTTCC ACCAGCGCCA CAAATCAGGG CGGCGGAATA
6781  TTCACTTTTG AGTCTTATCC CCTGACTATA TTTGCGCTCA AACTGAATAA AGCCATTCGC
6841  TTGTGCCTGA CTAGCGGGCT TTCACCGAAT GAACTGCAAA CTATCGTACG CAGTGACAAT
6901  GCACAAGGCA TCATCAACGA CTCCGTTCTG ACCAAAGTTT TCTATACTCT GTTCTACAGT
6961  CACCGTTATG CACTGAGCTT TGATGATGCA CAGGTACTGA ACGGATCGGT CATTAATCAA
7021  TATGCCCGAC GATGACAGTG TCAGTCATTT TAACCGTCTC TTTAATACCC CGCCGCTGAA
7081  AGGGAAAATC TTTGAAGCCG ACGGCAACAC GGTCAGCATT GATCCGGATG AAGAACAATC
7141  TACCTTTGCC CGTTCAGCCC TGATGCGTGG TCTGGGGATC AACAGTGGTG AACTGTATCA
7201  GTTAGGCAAA CTGGCGGGTG TATTGGACAC ACAAAATATC CTCACACTTT CTGTCCCTGT
7261  TATATCTTCA CTGTATCGCC TCACGTTACT GGCCCGTGCC CATCAGCTGA CGGTTAATGA
7321  ACTGTGTATG CTTTATGGTT TTTCGCCGTT CAATGGCAAA ACAACGGCTT CTTTGTCTTC
```

Fig.2.

```
 7381  CGGGGAGTTG TCACGGCTGG TTATCTGGTT GTATCAGGTG ACGCAGTGGC TGACTGAGGG
 7441  CGGAAATCAC CACTGAAGCG ATCTGGTTAT TATGTACGCC AGAGTTCAGC GGGAATATTT
 7501  CACCGGAAAT CAGTAATCTG CTTAATACTC TCCGACCCCG TATTAGTGAA GACATGGCAC
 7561  AAAGTAGTGA CCGGGAGCTT CAGGCTGAAA TTCTCGCGCC GTTTATTGCT GCAACGCTGC
 7621  ATCTGGCGTC ACCAGATATG GCGCGGTATA TCCTGTTGTG GACTGATAAC CTGCGGCCGG
 7681  GCGGCCTGAA TATCGCCGGA TTTATGATGC TGGTGCTGAA AGAGACGCTG AGTGATGAGG
 7741  AAACGACCCA ACTGGTTCAA TTCTGCCATG TAATGGCACA GTTATCGCTT TCCGTGCAGA
 7801  CACTGCGTCT CAGTGAAGCA GAGCTTTCTG TGCTGGTCAT TTCCGATTTT GTGGTACTGG
 7861  GTGCGAGAAG CCAACCGCCG GACAACACAA TATTGATACT CTGTTCTCAC TCTACCGATT
 7921  CCACCAGTGG ATTAATGGGC TGGGAAATCC CGGCTCTGAC ACGCTGGATA TGCTGCGCCA
 7981  AGCAGACACT CACGGGCGAC AGACTGGGCC TCCGTGATGG GGCTGGACAT CAGTATGGTA
 8041  ACGCAGGCCA TGGGTTCCCG CCGGCGTGAA CCAACTTCAG TGTTGGCAGG ATATCAACCC
 8101  CGTGTTGCAG TGGATACATG TGGCATCAGC ACTGCTCACT GATGCCGTCG GTTATCCGTA
 8161  CGCTGGTGAA TATCCGTTAC GTGACTGCAT TAAACAAAGC CGAGTCGAAT CTGCCTGCCT
 8221  GGGATAAGTG GCAGACGCTG GCAGAAAATA TGGCAGCCGG ACTGAGTACA CAACAGGCTC
 8281  AGACGCTGGC GGATTATACC GCAGAGCGCC TGAGTAACGT GTTGTGCAAT TGGTTTCTGG
 8341  CGAATATCCA GCCAGAAGGG GTGTCCCTGC ACAGCCGGGA TGACCTGTAC AGCTATTTCC
 8401  TGATTGATAA TCAGGTCTCT TCTGCCATAA AAACCACCCG ACTGGCAGAG GCCATTGCCG
 8461  GTATTCAGCT CTACATCAAC CGGGCGCTGA ACCGGATAGA GCCTAATGCC CGTGCCGATG
 8521  TGTCAACCCG CCAGTTTTTT ACCGACTGGA CGGTGAATAA CCGTTACAGC ACCTGGGGCG
 8581  GGGTGTCGCG GCTGGTTTAT TATCCGGAAA ATTACATTGA CCCGACCCAG CGTATCGGGC
 8641  AGACCCGGAT GATGGATGAA CTGCTGGAAG ATATCAGCCA GAGTCAGCTC AGCCGGGACA
 8701  CGGTGGAAGA GGCCTTTAAA ACTTACCTGA CCGCTTTGAA ACCGTGGCAG ACCTGAAAGT
 8761  TGTCAGCGCT ATCACCGACA ACGTCAACAG CAACACCGGA CTGACCTGGT TTGTCGGCCA
 8821  AACGCGGGAG AACCTGCCGG AATATTACTG GCGTAACGTG CATATATCAC GGATGCAGGC
 8881  GGGTGAACTG GCCGCCGATG CCTGGAAAGA TTGGACGAAG ATTGATACAG CGGTCAACCC
 8941  ATACAAGGAT GCAATACGTC CGGTCATATT CAGGGAACGT TTGCACCTTA TCGTGGGTAG
 9001  AAAAAGAGGA AGTGGCGAAA AATGGTACTG ATCCGGTGGA AACCTATGAC CGTTTTACTC
 9061  TGAAACTGGC GTTTCTGCGT CATGATGGCA GTTGGAGTGC CCCCTGGTCT TACGATATCA
 9121  CAACGCAGGT GGAGGCGGTC ACTGACAAAA AACCTGACAC TGAACGGCTG GCGCTGGCCG
 9181  CATCAGGCTT TCAGGGCGAG GATACTCTGC TGGTGTTTGT GTACAAAACC GGGGTGAGTT
 9241  ACCCGGATTT TGGCGACAAC AATAAAAATG TGGCAGGCAT GACCATTTAC GGCGATGGCT
 9301  CCTTCAAAAA GATGGAGAAC ACAGCACTCA GCGTTACAGC CAACTGAAAA ATACCTTTGA
 9361  TATCATTCAT ACTCAAGGCA ACGACTTGGT AAGAAAGGCC AGCTATCGTT TCGCGCAGGA
 9421  TTTTGAAGTG CCTGCCTCGT TGAATATGGG TTCTGCCATC GGTGATGATA GTCTGACGGT
 9481  GATGGAAAAC GGGAATATTC CGCAGATAAC CAGTAAATAC TCCAGCGATA ACCTTGCTAT
 9541  TACGCTACAT AACGCCGCTT TCACTGTCAG ATATGATGGC AGTGGCAATG TCATCAGAAA
 9601  CAAACAAATC AGCGCCATGA AACTGACGGG GTTGGATGAA AGTCCCAGTA CGGCAATGCA
 9661  TTTATCATCG CAAATACCGT TAAACATTAT GGCGGTTACT CTGATCTGGG GGGCCCGATC
 9721  ACCGTTTTTA TTAAAACGGA AAAACTATAT TGCATCAGTT CAAGGCCACT TGATGAACGC
 9781  AGATTACACT AGGCGTTTGA TTCTAACACC AGTTGAAAAT AATTATTATG CCAGATTGTT
 9841  CGAGTTTCCA TTTTCTCCAA ACACAATTTT AAACACCGTT TTCACGGTTG GTAGCAATAA
 9901  AACCAGTGAT TTTAAAAAGT GCAGTTATGC TGTTGATGGT AATAATTCTC AGGGCTTCCA
 9961  GATATTTAGT TCCTATCAAT CATCCGGCTG GCTGGATATT GACACAGGTA TTAACAATAC
10021  TGATGTCAAA ATTACGGTGG TAGCTGGCAG TAAAACCCAC ACCTTTACGG CCAGTGACCA
10081  TATTGCTTCC TTGCCGGCAA ACAGTTTTGA TGCTATGCCG TACACCTTTA AGCCACTGGA
10141  AATCGATGCT TCATCGTTGG CCTTTACCAA TAATATTGCT CCTCTGGATA TCGTTTTTGA
10201  GACCAAAGCC AAAGACGGGC GAGTGCTGGG TAAGATCAAG CAAACATTAT CGGTGAAACG
10261  GGTAAATTAT AATCCGGAAG ATATTCTGTT TCTGCGTGAA ACTCATTCGG GTGCCCAATA
10321  TATGCAGCTC GGGGTGTATC GTATTCGTCT TAATACCCTG CTGGCTTCTC AACTGGTATC
10381  CAGAGCAAAC ACGGGCATTG ATACTATCCT GACAATGGAA ACCCAGCGGT TACCGGAACC
10441  TCCGTTGGGA GAAGGCTTCT TTGCCAACTT TGTTCTGCCT AAATATGACC CTGCTGAACA
10501  TGGCGATGAG CGGTGGTTTA AAATCCATAT CGGGAATGTT GGCGGTAACA CGGGAAGGCA
10561  GCCTTATTAC AGCGGAATGT TATCCGATAC GTCGGAAACC AGTATGACAC TGTTTGTCCC
10621  TTATGCCGAA GGGTATTACA TGCATGAAGG TGTCAGATTG GGGGTTGGAT ACCAGAAAAT
10681  TACCTATGAC AACACTTGGG AATCTGCTTT CTTTTATTTT GATGAGACAA AACAGCAATT
10741  TGTATTAATT AACGATGCTG ATCATGATTC AGGAATGACG CAACAGGGGA TCGTGAAAAA
10801  TATCAAGAAA TACAAAGGAT TTTTGAATGT TTCTATCGCA ACGGGCTATT CCGCCCCGAT
10861  GGATTTCAAT AGTGCCAGCG CCCTCTATTA CTGGGAATGT TCTATTACAC CCCGATGATG
10921  TGCTTCCAGC GTTTGCTACA GGAAAAACAA TTCGACGAAG CCACACAATG GATAAACTAC
10981  GTCTATAATC CCGCCGGCTA TATCGTTAAC GGAGAAATCG CCCCCTGGAT CTGGAACTGC
11041  CGGCCGCTGG AAGAGACACT CCTGGAATGC CAATCCGTTG GATGCCATTG ATCCGGATGC
11101  CGTCGCACAA TATGACCCGA CACACTATAA AGTTGCCACC TTTATGCGCC TGTTGGATCA
11161  ACTTATTCTG CGCGGCGATA TGGCCTATCG CGAACTGACC CGCGATGCGT TGAATGAAGC
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Fig.2.

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11221  CAAGATGTGG TATGTGCGTG CTTTGGAATT GCTGGGTGAT GAGCCGGAGG ATTACGGCAG
11281  CCAACAGTGG GCCGCACCGT CTCTTTCCGT GGCGGGCAAC CACACTGTGC AAGCGGGCTA
11341  TCAACAAGAC CTTACGGCGC TAGACAACGG AGAAGGTTGC ACTCAACCCC GCAACGCTAA
11401  CTCGTTGGTG GTTTGGTCCT GCCGGAATAT AACCCGGAAT CAACCGATTA CTGGCAAACC
11461  TGCGTTTGCG CCTGGTTAAC CTGCGCCATA ATCCTTCCAT GACGGGCAAC CGTTATCGCT
11521  GGCGAATTAC GCGAGCCTAC GATCCGAAAG CGCTGCTCAC CAGTATGGTA CAGCCTTCTC
11581  AGGGCGGTAG TGCAGTGCTG CCCGGCACAT TGTCGTTATA CCGCTTCCCG GTGATGCTGG
11641  AGCGGGCCCG CAATCTGGTA GCGCAATTAA CCCAGTTCGG CACCTCTCTG CTCAGTATGG
11701  CAGAGCATGA TGATGCCGAT GAACTCACCA CGTTGCTACT ACAGCAGGGT ATGGAACTGG
11761  CGACACAGAG CATCCGTATT CAGCAACGAA CTGTCGATGA AGTGGATGCT GATATTGCTG
11821  TATTGGCAGA GAGCCGCCGC AGTGCACAAA ATCGTCTGGA AAAATACCAG CAGCTGTATG
11881  ACGAGGATAT CAACCACGGA GAACAGCGTG CGATGTCACT GTTTGATGCG GCGGCAGGTC
11941  AGTCTCTGGC CGGGCAGGCG CTCTCAGTAG CAGAAGGGGT GGCTGACTTA GTTCCAAACG
12001  TGTTCGGTTT CGCTTGTGGC GGCAGTCGTT GGGGGGCAGC ACTGCGTGCT TCCGCCTCCG
12061  TGATGTCGCT TTCTGCCACA GCTTCCCAAT ATTCCGCAGA CAAAATCAGC CGTTCGGAAG
12121  CCTACCGCCG CCGCCGTCAG GAGTGGGAAA TTCAGCGTGA TAATGCTGAC GGTGAAGTCA
12181  AACAAATGGA TGCCCAGCTG GAAAGCCTGA AAATACGCGG CGAAGCAGCA CAGATGCAGG
12241  TGGAATATCA GGAGACCCAG CAGGCCCATA CTCAGGCTCA GTTAGAGCTG TTACAGCGTA
12301  AATTCACAAA CAAAGCGCTT TACAGTTGGA TGCGCGGCAA GCTGAGTGCT ATCTATTACC
12361  AGTTCTTTGA CCTGACCCAG TCCTTCTGCC TGATGGCACA GGAAGCGCTG CGCCGCGAGC
12421  TGACCGACAA CGGTGTTACC TTTATCCGGG GTGGGGCCTG GAACGGTACG ACTGCGGGTT
12481  TGATGGCGGG TGAAACGTTG CTGCTGAATC TGGCAGAAAT GGAAAAAGTC TGGCTGGAGC
12541  GTGATGAGCG GGCACTGGAA GTGACCCGTA CCGTCTCGTT GGCACAGTTC TATCAGGCCT
12601  TATCATCAGA CAACTTTAAT CTGACCGAAA AACTCACGCA ATTCCTGCGT GAAGGGAAAG
12661  GCAACGTAGG AGCTTCCGGC AATGAATTAA AACTCAGTAA CCGCCAGATA GAAGCCTCAG
12721  TGCGATTGTC TGATTTGAAA ATTTTCAGCG ATACCCCGGA AAGCTTTGGC AATACCCGTC
12781  AGTTGAAACA AGTGAGTGTC ACCTTGCCGG CGCTGGTTGG TCCGTATGAA GATATCCGGG
12841  CGGTGCTGAA TTACGGCGGC AGCATCGTCA TGCCACGCGG TTGCAGTGCT ATTGCTCTCT
12901  CCCACGGCGT GAATGACAGT GGTCAATTTA TGCTGGATTT CAACGATTCC CGTTATCTGC
12961  CGTTTGAAGG TATTTCCGTG AATGACAGCG GTAGCCTGAC GTTGAGTTTC CCGGATGCGA
13021  CTGATCGACA GAAAGCGCTG CTGGAGAGCC TGAGCGATAT CATTCTGCAT ATCCGCTATA
13081  CCATTCGTTC TTAATTAAAA CATTGTGATA GGCAGGCTCC TGAGGGAGCC TGTTTAAGGA
13141  GTTTTTATGC AGGGTTCAAC ACCTTTGAAA CTTGAAATAC CGTCATTGCC CTCTGGGGGC
13201  GGATCACTAA AAGGAATGGG AGAAGCACTC AATGCCGTCG GAGCGGAAGG GGAGCGTCAT
13261  TTTCACTGCC CTTGCCGATC TCTGTCCGGC GTGGTCTGGT GCCGGTGCTA TCACTGAATT
13321  ACAGCAGTAC TGCTGGCAAT GGGTCATTCG GGATGGGGTG GCAATGTGGG GTTGGTTTTA
13381  TCAGCCTGCG TACCGCCAAG GGCGTTCCGC ACTATACGGG ACAAGATGAG TATCTCGGGC
13441  CGGATGGGGA AGTGTTGAGT ATTGTGCCGG ACAGCCAAGG GCAACCAGAG CAACGCACCG
13501  CAACCTCACT GTTGGGGACG GTTCTGACAC AGCCGCCTAC TGTTACCCGC TATCAGTCCC
13561  GCGTGGCAGA AAAAATCGTT CGTTTAGAAC ACTGGCAGCC ACAGCAGAGA CGTGAGGAAG
13621  AGACGTCTTT TTGGGTACTT TTTACTGCGG ATGGTTTAGT GCACCTATTC GGTAAGCATC
13681  ATCATGCACG TATTGCTGAC CCGCAGGATG AAACCAGAAT TGCCCGCTGG CTGATGGAGG
13741  AAACCGTCAC GCATACCGGG GAACATATTT ACTATCACTA TCGGGCAGAA GACGATCTTG
13801  ACTGTGATGA GCATGAACTT GCTCAGCATT CAGGTGTTAC GGCCCACCGT TATCCTGGCA
13861  AGTCCACTAT GGCAATACTC AGCCGGAAAC CGCTTTTTTC GCGGTAAAAT CAGGTATCCC
13921  TGTTGATAAT GACTGGTTGT TTCATCTGGT ATTTGATTAC GGTGAGCGCT TATCTTCGCT
13981  GAACTCCGTA CCCGAATTCA ATGTGTCAGA AAACAATGTG TCTGAAAACA ATGTGTCTGA
14041  AAAATGGCGT TGTCGTCCGG ACAGTTTCTC CCGCTATGAA TATGGGTTTG AAATTCGAAC
14101  CCGTCGCTTG TGTCGCCAAG TTCTGATGTT TCATCAGCTG AAAGCGCTGG CAGGGGAAAA
14161  GGTTGCAGAA GAAACACCGG CGCTGGTTTC CCGTCTTATT CTGGATTATG ACCTGAACAA
14221  CAAGGTTTCC TTGCTGCAAA CGGCCCGCAG ACTGGCCCAT GAAACGGACG GTACGCCAGT
14281  GATGATGTCC CCGCTGGAAA TGGATTATCA ACGTGTTAAT CATGGCGTGA ATCTGAACTG
14341  GCAGTCCATG CCGCAGTTAG AAAAAATGAA CACGTTGCAG CCATACCAAT TGGTTGATTT
14401  ATATGGAGAA GGAATTTCCG GCGTTACTTT ATCAGGATAC TCAGAAAGCC TGGTGGTACC
14461  GTGCTCCGGT ACGGGATATC ACTGCCGAAG GAACGAATGC GGTTACCTAT GAGGAGGCGA
14521  AACCACTGCC ACATATTCCG GCACAACAGG AAAGCGCGAT GTTGTTGGAC ATCAATGGTG
14581  ACGGGCGTCT GGATTGGGTG ATTACGGCAT CAGGGTTACG GGGCTACCAC ACCATGTCAC
14641  CGGAAGGTGA ATGGACACCC TTTATTCCAT TATCCGCTGT GCCAATGGAA TATTTCCATC
14701  CGCAGGCAAA ACTGGCTGAT ATTGATGGGG CTGGGCTGCC TGACTTAGCG CTTATCGGGC
14761  CAAATAGTGT ACGTGTCTGG TCAAATAATC CGGCAGGATG GGATCGCGCT CAGGATGTTA
14821  TTCATTTGTC AAATAAGCCA CTGCCGGTTC CCGGCAAAAA TAAGCGTCAT CTTGTCGCAT
14881  TCAGTGATAT GACAGGCTCC GGGCAATCAC ATCTGGTGGA AGTTACGGCA AATAGCGTGC
14941  GCTACTGGCC GAACCTGGGG CATGGAAAAT TTGGTGAGCC TCTGATGATA ACAGGCTTCC
15001  AAATTACGGG GAAACGTTTA ACCCCCACAG ACTGTATATG GTAGACCTAA ATGGCTCAGG
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Fig.2.

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15061  CACCACCCGA TTTTATTTAT GCCCGCAATA CTTACCTTGA ACTCTATGCC AATGAAAGCG
15121  GCAATCATTC TGCTGAACCT CAGCGTATTG ATCTGCCGGA TGGGGTACGT TTTGATGATA
15181  CTTGTCGGTT ACAAATAGCG GATACACAAG GATTAGGGAC TGCCAGCATT ATTTTGACGA
15241  TCCCCCATAT GAAGGTGCAG CACTGGCGAT TGGATATGAC CATATTCAAG CCTTGGCTGC
15301  TGAATGCCGT CAATAACAAT ATGGGAACAG AAACCACGCT GTATTATCGC AGCTCTGCCC
15361  AGTTCTGGCT GGATGAGAAA TTACAGGCTT CTGAATCCGG GATGACGGTG GTCAGCTACT
15421  TACCGTTCCC GGTGCATGTG TTGTGGCGCA CGGAAGTGCT GGATGAAATT TCCGGTAACC
15481  GATTGACCAG CCATTATCAT TACTCACATG GTGCCTGGGA TGGTCTGGAA CGGGAGTTTC
15541  GTGGTTTTGG GCGGGTGACG CAAACTGATA TTGATTCACG GGCGAGTGCG ACACAGGGGA
15601  CACATGCTGA ACCACCGGCA CCTTCGCGCA CGGTTAATTG GTACGGCACT GGCGTACGGG
15661  AAGTCGATAT TCTTCTGCCC ACGGAATATT GGCAGGGGGA TCAACAGGCA TTTCCCCATT
15721  TTACCCCACG CTTTACCCGT TATGACGAAA AATCCGGTGG TGATATGACG GTCACGCCGA
15781  GCGAACAGGA AGAATACTGG TTACATCGAG CCTTAAAAGG ACAACGTTTA CGCAGTGAGC
15841  TGTATGGGGA TGATGATTCT ATACTGGCCG GTACGCCTTA TTCAGTGGAT GAATCCCGCA
15901  CCCAAGTACG TTTGTTACCG GTGATGGTAT CGGACGTGCC TGCGGTACTG GTTTCGGTGG
15961  CCGAATCCCG CCAATACCGA TATGAAGGGG TTGTTACCGA TTCCACAGTG CAGCCAAAAG
16021  ATTGTCCTTA AATATGATGC GTTAGGATTT CCGCAGGACA ATCTTGAGAT TGCCTATTCG
16081  AGACGTCCAC AGCCTGAGTT CTCGCCTTAT CCGGATACCC TGCCCGAAAC ACTTTTCACC
16141  AGCAGTTTCG ACGAACAGCA GATGTTCCTT CGTCTGACAC GCCAGCGTTT TTCTTATCAC
16201  CATCTGAATC ATGATGATAA TACGTGGATC ACAGGGCTTA TGGATACCTC ACGCAGTGAC
16261  GCACGTATTT ATCAAGCCGA TAAAGTGCCG GACGGTGGAT TTTCCCTTGA ATGGTTTTCT
16321  GCCACAGGTG CAGGAGCATT GTTGTTGCCT GATGCCGCAG CCGATTATCT GGGACATCAG
16381  CGTGTAGCAT ATACCGGTCC AGAAGAGCAA CCCGCTATTC CTCCGCTGGT GGCATACATT
16441  GAAACCGCAG AGTTTGATGA ACGATCGTTG GCGGCTTTTG AGGAGGTGAT GGATGAGCAG
16501  GAGCTGACAA AACAGCTGAA TGATGCGGGC TGGAATACGG CAAAAGTGCC GTTCAGTGAA
16561  AAGACAGATT TCCATGTCTG GGTGGGACAA AAGGAATTTA CAGAATATGC CGGTGCAGAC
16621  GGATTCTATC GGCCATTGGT GCAACGGGAA ACCAAGCTTA CAGGTCAAAC GACAGTGACG
16681  TGGGATAGCC ATTACTGTGT TATCACCGCA ACAGAGGATG CGGCTGGCCT GCGTATGCAA
16741  GCGCATTACG ATTATCGATT TATGGTTGCG GATAACACCA CAGATATCAA TGATAACTAT
16801  CACACCGTGA CGTTTGATGC ACTGGGGACG GTAACCAGCT TCCGTTTCTG GGGGACTGAA
16861  AACGGTGAAA AACAAGGATA TACCCCTGCG GAAAATGAAA CTGTCCCCTT TATTGTCCCC
16921  ACAACGGTGG ATGATGCTCT GGCATTGAAA CCCGGCATAC CTGTTGCAGG GCTGATGGTT
16981  TATGCCCCTC TGAGCTGGAT GGTTCAGGCC AGCTTTTCTA ATGATGGGGA GCTTTATGGA
17041  GAGCTGAAAC CGGCTGGGAT CATCACTGAA GATGGTTATC TCCTGTCGCT TGCTTTTCGC
17101  CGCTGGCATC AAAATAACCC TGCCGCTGCC ATGCCAAAGC AAGTCAATTC ACAGAACCCA
17161  CCCCATGTAC TGAGTGTGAT CACCGACCGC TATGATGCCG ATCCGGAACA ACAATTACGT
17221  CAAACGTTTA CGTTTAGTGA TGGTTTTGGG CGAAACCTTA CAAACAGCCG TACGCCATGA
17281  AAGTGGTGAA GCCTGGGTAC CTGATGAGTA TGGAGCCAAT GTGGCTGAAA ATCAAGGCGC
17341  CCCTGAAACG GGCGATTACA AATTTCCCGT TGGGCAATTT CCCGGACGTA CAGAATATTA
17401  ACGGGAAAAG GCAAAGCCCC TGCGTTACGT TTCAAACCGT ATTCCTGAAA TAATTTGGGC
17461  AACTATGTCA AGTTGACCAA AAAATGCCCG GCAGGATATG TATGCCGATA CCCATTACTA
17521  TGATCCGTTG GGGCGTGAAT ATCAGGTTAT CACGCCAAAG GCGGGTTGCG TCGATCCTTA
17581  TTCACTCCCT GGTTTGTGGT GAATGAAGTT GAAAATGACA CTCCCGGTGA ATGACAGCAT
17641  AAAGCTCAGT GATGCCTGTT CACTGAACAG ACATCACTCC ATTTAGGAAT GAATCATGAA
17701  GAATTTCGTT CACAGCAATA CGCCATCCGT CACCGTACTG GACAACCGTG GTCAGACAGT
17761  ACGCGAAATA GCCTGGTATC GGCACCCCGA TACACCTCAG GTAACCGATG AACGCATCAC
17821  CGGTTATCAA TATGATGCTC AAGGATCTCT GACTCAGAGT ATTGATCCGC GATTTTATGA
17881  ACGCCAGCAG ACAGCGAGTG ACAAGAACGC CATTACACCC AATCTTATTC TCTTGTCATC
17941  ACTCAGTAAG AAGGCATTGC GTACGCAAAG TGTGGATGCC GGAACCCGTG TCGCCCTGCA
18001  TGATGTTGCC GGGCGTCCCG TTTTAGCTGT CAGCGCCAAT GGCGTTAGCC GAACGTTTCA
18061  GTATGAAAGT GATAACCTTC CGGGACGATT GCTAACGATT ACCGAGCAGG TAAAAGGAGA
18121  GAACGCCTGT ATCACGGAGC GATTGATTTG GTCAGGAAAT ACGCCGGCAG AAAAAGGCAA
18181  TAATTTGGCC GGCCAGTGCG TGGTCCATTA TGATCCCACC GGAATGAATC AAACCAACAG
18241  CATATTGTTA ACCAGCATAC CCTTGTCCAT CACACAGCAA TTAGTGAAAG ATGACAGCGA
18301  AGCCGATTGG CACGGTATGG ATGAATTTGG CTGGAAAAAC GCGCTGGCGC CGGAAAGCTT
18361  CACTTCTGTC AGCACAACGG ATGCTACCGG CACGGTATTA ACGAGTACAG ATGCTGCCGG
18421  AAACAAGCAA CGTATCGCCT ATGATGTGGC CGGTCTGCTT CAAGGCAGTT GGTTGGCGCT
18481  GAAGGGGAAA CAAGAACAAG TTATCGTGAA ATCCCTGACC TATTCGGCTG CCAGCCAGAA
18541  GCTACGGGAG GAACATGGTA ACGGGATAGT GACTACATAT ACCTATGAAC CCGAGACGCA
18601  ACGAGTTATT GGCATAAAAA CAGAACGTCC TTCCGGTCAT GCCGCTGGGG AGAAAATTTT
18661  ACAAAACCTG CGTTATGAAT ATGATCCTGT CGGAAATGTG CTGAAATCAA CTAATGATGC
18721  TGAAATTACC CGCTTTTGGC GCAACCAGAA AATTGTACCG GAAAATACTT ACACCTATGA
18781  CAGCCTGTAC CAGCTGGTTT CCGTCACTGG GCGTGAAATG GCGAATATTG GCCGACAAAA
18841  AAACCAGTTA CCCATCCCCG CTCTGATTGA TAACAATACT TATACGAATT ACTCTCGCAC
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Fig.2.

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18901  TTACGACTAT GATCGTGGGG GAATCTGACC AGAATCGCAT AATTCACGAT CACCGGTAAT
18961  AACTATACAA CGAACATGAC CGTTTCAGAT CACAGCAACC GGGCTGTACT GGAAGAGCTG
19021  GCGCAAGATC CCACTCAGGT GGATATGTTG TTCACCCCCG GCGGGCATCA GACCCGGCTT
19081  GTTCCCGGTC AGGATCTTTT CTGGACACCC CGTGACGAAT TGCAACAAGT GATATTGGTC
19141  AATAGGGAAA ATACGACGCC TGATCAGGAA TTCTACCGTT ATGATGCAGA CAGTCAGCGT
19201  GTCATTAAGA CTCATATTCA GAAGACAGGT AACAGTGAGC AAATACAGCG AACATTATAT
19261  TTGCCAGAGC TGGAATGGCG CACGACATAT AGCGGCAATA CATTAAAAGA GTTTTTGCAG
19321  GTCATCACTG TCGGTGAAGC GGGTCAGGCA CAAGTGCGGG TGCTGCATTG GGAAACAGGC
19381  AAACCGGCGG ATATCAGCAA TGATCAGCTG CGCTACAGTT ATGGCAACCT GATTGGCAGT
19441  AGCGGGCTGG AATTGGGACA GTGACGGGCA GATCATTAGT CAGGAAGAAT ATTACCCCTA
19501  TGGGGGAACC GCCGTGTGGG CACCCGAAAT CAGTCAGAAG CTGATTACAC AAGCCGGCGT
19561  TATTCTGGCA AAGAGCGGGA TGCAACAGGG TTGTATTACT ACGGCTATCG TTATTATCAA
19621  TCGTGGACAG GGCGATGGTT GAGTGTAGAT CCTGCCGGTG AGGCCGATGG TCTCAATTTG
19681  TTCCGAATGT GCAGGAATAA CCCCATCGTT TTTTCTGATT CTGATGGTCG TTTCCCCGGT
19741  CAGGGTGTCC TTGCCTGGAT AGGGAAAAAA GCGTATCGAA AGGCAGTCAA CATCACGACA
19801  GAACACCTGC TTGAACAAGG CGCTTCCTTT GATACGTTCT TGAAATTAAA CCGAGGATTG
19861  CGAACGTTTG TTTTGGGTGT GGGGGTACAA GTCTGGGGGT GAAGCGGCCA CGATTGCAGG
19921  AGCGTCGCCT TGGGGGATCG TCGGGGCTGC CATTGGTGGT TTTGTCTCCG GGGCGGTGAT
19981  GGGGTTTTTC GCGAACAACA TCTCAGAAAA AATTGGGGAA GTTTTAAGTT ATCTGACGCG
20041  TAAACGTTCT GCTCCTGTTC AGGTAGGCGC TTTTGTTGTC ACATCGCTTG TGACGTCTGC
20101  ACTATTTAAC AGCTCTTCGA CAGGTACCGC CATTTCCGCA GCAACAGCGG TCACCGTTGG
20161  AGGATTAATG GCTTTAGCCG GAGAACATAA CACGGGCATG GCTATCAGTA TTGCCACACC
20221  CGCCGGACAA AGTACGCTGG ATACGCTCAG GCCCGGTAAT GTCAGCGCGC CAGAGCGGTT
20281  AGGGCACTAT CAGGCGCAAT TATTGGCGGC ATATTACTTG GCCGCCATCA GGGAAGTTCT
20341  GAGCTGGGTG AACGGGCAGC GATTGGTGCT ATGTATGGTG CTCGATGGGG AAGGATCATT
20401  GGTAATCTAT GGGATGGCCC TTATCGGTTT ATCGGCAGGT TACTGCTCAG AAGAGGCATT
20461  AGCTCTGCCA TTTCCCACGC TGTCAGTTCC AGGAGCTGGT TTGGCCGAAT GATAGGAGAA
20521  AGTGTCGGGA GAAATATTTC TGAAGTATTA TTACCTTATA GCCGTACACC CGGTGAATGG
20581  GTTGGTGCAG CCATTGGCGG GACAGCCGCG GCCGCTCATC ATGCCGTTGG AGGGGAAGTT
20641  GCCAATGCCG CTAGCCGGGT TACCTGGAGC GGCTTTAAGC GGGCTTTTAA TAACTTCTTC
20701  TTTAACGCCT CTGCACGTCA TAATGAATCC GAAGCATAAC AATCATGTTC ATTCCCACTT
20761  TGTCATGGAT GACAAGGTGG GTTTTTCGGA TGTGTGGACA GAGACCCGTA CAGGGTCTCT
20821  GTCCAGTTAA TTTTTGGATC AAGAACGAAT GGTGTAACGG ATATGCAAAA TGATATCGCT
20881  CAGGCTGAGC AATAAGCTTT TCTGTTTACC ACTGATACCG GGAAAACTGA GGGTTAATGT
20941  GCCTGTATCG GCCACAGGAA GCCCTTCAAA TGGCAGGTAC TTAGCATCAT TGAAATCCAT
21001  CTGGAATTGA CCACTGTCAT TCATGCCATG TGAGATCACA ATCGCTTTGC AGCCACGTGG
21061  CATCATTGTA CTGCCGCCAT AACTCAGTAT TGCCCGGACA TCCTGATAAG GCCCTAAAAG
21121  GGCAGGTAAC GTCACACTGA TTTGTTTGAT ACGGCGTGTA TTACCTAAAC CGTCAGGATA
21181  ATCGGTAGCA ATATTCAGAT CCGATAATTT GAGGCTGGCT TGCAGTTGTG TCCCTTCGAC
21241  GTTCAAACCG TTAAGCGTTG TGCCTGCACT GCCTTCACCT GCATTGACTA ACTCAGTCAC
21301  TTTATCTTTT AAAATGAAAC TATTTTCTGT CAGACCAGCA TACACTTCAG CCAGAGAAAC
21361  GGTTCTGGTG ACCTCCAGTG CCCGTTCATC TTTTTCCAAA TAGCTTTTTT CCATCTGTGC
21421  TAAATTCAGC ATCAGGGTTT CACCCGCTAA TAAACCCGCA TAAGTCCCAT GCCAAGCACC
21481  TGGTTTAATA AAGTGTGCTG CCGCATTATT CAATTCATAC TGATAAGTTT GCTCTGCCAT
21541  TAAACAGAGT GAGACCGCCA AATCATAAAA CTGATAATAA ATAGCGGACA ACGTTCCACG
21601  GAGCCAGTTG TATAGCGCTG CATTACTGAA TTTACTTTGC AGAAAGGCTA ACTGCGCCTG
21661  AGTTTGTGCC TGCTGAGTTT CCAGATAGTT TTTTTGTAAT ACTGCCGCTT CACGACGTAC
21721  AGCCAGCGTC GCTAATTGAG CATCAATTTG TTTTATCTCA GCTTCCGCAT TATTGCGCTG
21781  AATTTCCCAC TCTTGCCGAC GGCGACGGTA TATTTCTGAT TGGCTGATTT TGTCTGCGGC
21841  AATACGTGTT GCTGACGCAG AAATTTCGAT ACCAATCGCA CTGGCATTGA AAAGCGCCCC
21901  AAAACGGGAA CCTCCCACAG CAAAACCGTA AATATTGGGG ACGAGATCTG CCGCGGCGGC
21961  GGCCATATGC AGGGCTGTGC CGCTGGTGCT CAAGACCGAT GAAGAGAGGT AAAGATCCAT
22021  CGCTTGTTTT TCACCAGCGT TAACATCTTC GTCGTACAGC GTATTGAAAC TGTCAAAACG
22081  AGACTGTGCA CCATGACGGC TTTCTTGAAG CGCCAATTTA TCAGCATCAA TTTCAGCCAT
22141  GACCTTATCC TGCATTTTAA TACTTTGCAG GGCTAACTCA CTGCCTTGAG TTTGCAGTAT
22201  TTCAGCCAAG GCTTCTGCAT CCTGCCGTTC AGTAATGCTG AGCAGGGTAT TGCCAAATTG
22261  TATCAACTGG CTTACCCCCC ACTTGGCATT TTCCAGAATC ACCGGAAAAC GGTACATCGG
22321  CATCACTGCA TGAGGTAAAT CGCCGCCGCC TTGTGAAGCA GTGATGGCAG CACTGAGTAA
22381  CATGGACGGA TCTGCGGGCG TGGCATAGAG AGATAATGAC AGTGGCTGAC CGTCGATTGT
22441  CAGGTTATGG CGTAAGTTAT AGAGGCGTTG CGTCAATGTC TGCCAGTAAC CTTGCAGTTT
22501  TTTATTAATT TGAGGGAGGA ACAATGCGGT TAACGAAATT TGCCGTACGT TTCGTGGGTA
22561  ATGCAGCGCG CTGACGCAGT TGCAGCATTT TATGTTGATA ATGATGCCGC ATTGTTTGGC
22621  TGGCAGCTTC TTCCAGCCGT GGCTCTGACC AATCGTTATC CAATGAAAAA TAAGGCTCAT
22681  CACCCAATAA AGTGAGCGCC TGTACATACC ACATTTTAGC TTCGTTTAAG GTATCACGTT
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Fig.2.

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22741 CAAGCTGGCG ATAGGCGCTA TCTCCGCGGG TAATCAACAA ATCCAGCATT TTCATAAAGG
22801 TAGCCACTTT ATAGTGCATC GGATCATGCT GGGCAACGGC GTCCGGATCG ACCGAATCCA
22861 GCGGATTGGC ATTCCAGGAC GTATCTTCCT CCAATGGGCG GACGTTCCAG TAATAATCCT
22921 GCATTTCACC CTGAACCGAA TATCCGGTCG GGTTCAGATA TAGCGCAGCC AGCGTGTCGA
22981 TCCGGTAAAA TCTGCTCTTG CAATAAGCGC TGGAATACCA TCATGGGCGT TGTAATAGAA
23041 CAATCCCAAG AAATAGATTG CATTGGCGCC GTTTGAAATC CATGGGTTCA GTGTTATTTT
23101 TCATGACACG ACTTGAATAC CCCTTTTATA TTTTTTGATA TTTTTTACTA TCCCCTGTTG
23161 TGTCATTCCC GAATCATGAT CGGCATCATT AGTGAATATA AATTGATTTT TCGTCTCATC
23221 AAAATAAAAG AAAGCAGATT CCCAGGATTT GTCATAGATA ATTTTTTTGT ACCCAACCCC
23281 TAATCTGACA CCTTCACGTA TGTAATATCC TTTAGCATAG GGAACAAAGA GCGTTACTGT
23341 GGTTTCAATA TCAGATAACA TTCCTTCGTA ATAAGGTTGT CTGGCAGAAT TGCCATCAAT
23401 ATTCCCAATA TGGATCTTAA ACCAACGTTC ATCACCATGC TCCTCTTTAT TGTAGGGGGG
23461 CAACTTAAAT GTCGCATAAA ACCCTTCACC TAATTGCGGC TCTGGTAAAT TTTGCGTTTC
23521 CATACTTAAA ACATTATCAA TACCAATATT GGCTCTTTCA GCTAATTTTC TGGAAAATAA
23581 AGTATTTAAC CGGGTTCTGT AAGGGCCAAT CTGCATATAT TGTGTGCCTG ATGGCATTTT
23641 ATGCAGTGAT ATAACGTTAC TTGTATCTTT GGATTTTAGT TTTATATGAA TTGGCGATTC
23701 AATAACAATA TCGTTATAAC CGCCGTCGGG TTGCTTAATA ATAAACTCGC TCACCAGAGG
23761 AATATCATAG CCTTCAATAT CAACTTTTAC TTGATTAAAA TCATATACCA TAGGGTCAGA
23821 TTCGTGTGAA GGTTTAGATG CCACATGGTC TTCAGCATTT AACTCCACTA GAATATCAGA
23881 GCCATTTTTT AATAAAAAAC TAATGTTTTT ATCTTGGATC TGTTCGATCA TAGATGAAGC
23941 AAGTTTTATT ATCTGTGGCT GGTTGAACAT AAATACACCC ATGGATCCTC GCGAAGGAAC
24001 AGTGCCGCAA TATTTCCCAT GTTATTAATG ATTGAAACAT CATTAGTAAA TGATTCACAT
24061 ATAGTATGCC ATACTCCTGT GTTATCTTTC CAATCTAATA CTATGTTAGT ATCAAGTTTG
24121 AATTCAGCAT CATCTGATTC ATAATCATAA TTTATACCAA CTCCAATTTC TGATTTTCTA
24181 GGAATTTTTT CCTTGGTTCT TAGATGCATT AACACTCTAA AATATTCGGC ATTTTTAAGA
24241 TCGATGGAAA TAATAAAATC CAAAGTTCCA TAATGAAAAA CTTCTTCTTC TTTTCCAAGC
24301 ATTTCATCAT GTCTATCATA ATCAAATAAA ATAACCGTTT CATCTTCTAC CATCGATAAC
24361 AGGTATTTAA CCTCATCATT ATATATATTG CCTTTTGAAA AATTAATTTC CATTGAAGGA
24421 TTGAACGTTA AATTAATATG ACCATTTCCT GGTGATATAT ACGAGAGATC AAAAATATTT
24481 CCGGTAAAAC TGGCTAATTT ATTTTTTGTG GTTATAGATT CCTTATATTC GGCCAAATAA
24541 TCTGTAGCAA ATTGATTGTT GACTTTGTAT TCTGTCCTGG TATCAAGTTC TGATAATGTG
24601 CTCTTAACAA TGGCGTCTAA ATCATTTTCT GTGAGAATGG ATAATGTCAT ATCAGGGTTA
24661 ATGGTCATCC CTTCTCTTGC AGGAAGACTA TTAAAAGAAT AATTGTCTTT TTTCTCATGG
24721 AAATAAACAA TAATGACGTC TTTTTCATAA TCAGAAGAAC AATACATACC AATGCTGGCT
24781 TTTTTATTGA TCAGGTTTTC TATTTTATCA GTCACATTAA AATTAAACGG TGAGCTCCAG
24841 CTGCCATCAT AACGAATATG TGACAGTTTT AATATATAAT CAGTGATATC TATCTTGCCA
24901 TCTTCACTTT CATTTTTCAG CTCTTTTTGT TCCAGCCACA GTAAATACAA ACGAGACTTG
24961 TAAATAACAG GTCTGATATT TTCCTGCCAT ACATTGATGG GTATTTCAAT TTTTTTCCAT
25021 TCTCCCCAGG CATTGGCAGC AAATTGACCG TGCTGGCACT TTTGGTGATC GACATTGCGC
25081 CAATAATATA TTCTGGGTTC TGTCTGGCTA TAACCAATTA AATAAGTGAG CCCCTCATTG
25141 ACATTAATAC TGTCATGATA TCCGCTAATC ACCTGCAAGT TAGCGACATC TTCAAATGCG
25201 GTCAGATAAT TTTTAAAGCT ATCTTCAACG GTATCGATAT TTAACTGACT TTGGGAAAGT
25261 TGCTGTAACA GGTTGTTCAT CATACCTGTC TGACCAATAC GAATCGTGGG GTCGATATAG
25321 TTTTCCGGAT AATAGGCCAG TTCAGATACG CCGGCCCAGG TGCTATACCG TCGATTGTAG
25381 GTTTCCCAGT CGCAGAAGAA CTGACGGGTT TTCACTGGCT TTGATACTTT TCCTTCAACA
25441 TTATTCAACG CCCGGTTGAC ATATAACTGA ATGCTGGCAA TGGCTTCTGC CACACGGGTG
25501 GTTTTCACTT GGGCAGAAAC TTGGTTATCA ATCAGCAGAT AGCTGTACAA CTCATCCCGG
25561 CTCTTAATCT GTTGAGGTGC ACCATTTTTG ATGTAGTAAG CACTGGCCGC TGTCGTCGTG
25621 GCTTCATCCA GCCATGCCTG AAGCTGGTCG GATTGTTGAC TGTTCAGTCC CGCCTGCAAC
25681 AAAGTACTGG CGGCTTGCCA ATCATCAAAT GTTGGCATCG GGGTTTCCGG TTCACCGACA
25741 TATTTTAATT TTATGAGTGC AGCAACACCA TCCGGGGTAA TACCCAATGT AGCAGCGACA
25801 TCCAGCCATT GCAGAGTGAC ATCTATAAGT TCTCCAGTTG GTAAAGGTAT TCACTCCCAA
25861 ACCGGTCTGT TGCAATGCTT GTGTCACAAC CTGAGCATCA AAATTTTAAC GCCACCGCCA
25921 AATTGTTCGG CAGTCAACGC TCCTAAGTTC CAAATGCTGT TAAGATTCTG TCGCGTAGCT
25981 TCACAACGCA TGATCACAGC ATGGAAGCGG GTCAGCGCTT GCAAAGTGGG GAGATCATGT
26041 TGCAGTGCTG TGGTTTCTGA TTGGAATTTC TCCGGTTTTG TCACCAACAG GGTCAGTTCG
26101 TTTTCGCTGA GTCCAATATT GCGCACAATC AGAGAAAGTT GCCCCAGTAC CTGACAAAAA
26161 GCCACCATGT TGCTGGTTTC ATTCTCTGAG CGATCACGGT TAGCCGCAAT AATCATGAAA
26221 TCATCGAATG TCAGTCCTTG TGGTTTTATC TGATTAATCC ACAGCAAAAT AGTTTCTGCT
26281 GTTTTGGCTG AATCCATTTG AATGCTGGCA GCAATCAGCG GGGCAGCTGC ACGGATCAGT
26341 TCGTCATCAC CGAGTGAAAG TGTTGATAAT CCATTACTTA GTGTCGTGAT AAGGTTTTCA
26401 ATATCCGGCG TAAGGACAGT GCTGTAATTA TCCGTGGTCA TCAGAAACAC ATCACTGACA
26461 GACCATTTCT GTGTTGTCAG CCACTGGGTG CATTGGAACA GAAAGCTGAT TAATTGCGTT
26521 AATGCTGTAT CAGAAAAAAG GGCAATTTTC GTGTTCACAT AGGGAGAAAC CGACAACAAC
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Fig.2.

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26581 ATGGATAATT CATTCACTGT CAGATGATGA ATGTCTGCCA GCAGACGAAC GCGATAAAGC
26641 AGAGACAGGT TCTCGATGGA ACACATAAAT TCTGGATTTG TTCCGCCATT AGCCAGTTTC
26701 CATAATGTAT ACAGTTCAGT ATCATTCACT CTGAAAGCAC GTTTCATTAT TCCCAAATAA
26761 AAATGGTTTT TTGATTCACC GGGGGTTAAA TCCAGTTTGG TATTATCAGC AGAAAACTCT
26821 TGGCCATTTA ATAGCGGTGT ATTGAACAGC ATTGTAAAAT GACTGGGTTG TTGTTTAGTG
26881 GAATATTGGC TGATATCTGA ATGACACAAT ACCAGCGCAT CGCTGACGCT AATATTATAG
26941 TGCTGCATAT AATATTGAAC ATAAAACAGC TTACCCAACA CATTGCTGTC AATGGTTAAG
27001 TCATCATAAA TACTTTCTAT TACTTGCCAG ATATCTTCTG GAGATATGCC TGTGGCTTTA
27061 TACAAACGAA TCGCTTTATT CAGCTTTAAC AGGAATATAT CACCGGGAAC TCCATCATTT
27121 TAAAGTGTGC ATTGGCATTG ATAGCATCCG ACGGATTTGG TTAACTCGCC ATAAGCGGAG
27181 TGTTATACCG TTGGTGATTT GCTCTGTCGT CAATTTAATG GGAATACTGT AATGGGTATT
27241 AGCAATGGGG ACGAAATTTT TATCTTGGTA TATATATTCT TTATCTCCAT TCTGGAGACG
27301 AAAATCCAAG TGGTCAGGTT CTGTTTTTTT TACACTGAAA TTATATTTGT ATTCATTTTC
27361 TTTGATTGGA ATTAGCTCTG CATAGTTTAA ATGTGAATCG TAGAAATCTT TGCGGGTTCG
27421 CTTAATCAAT CTTGCCGTTG CCGTATCATT CCCGTCATTG ACCAATGTTA TCAGTTGCTC
27481 ATTCTTATAC TGTTGATTTG TATTTTTCTT ACCGAAGGAG AGATTGACAA ATAAACTGAG
27541 TTCATCATAA GACAAATCGT AGTAGCGAGC CAAAGAAGCA TAACTCTTAA AAATCAGTAC
27601 ATCATCTGTA CCGAAATTTT TCTTCATCAG TTCTGTTGAA TTTTCCGGTG TAATTTCTTC
27661 TACAAGGATT TGATACAATT CAGGCGATAT ATCAGTCTTA ATAGCCAGTA GCGATGTTGG
27721 GTCCATTAAT TCCGCTACGT CTGTATTACG GCTAAATGCG GTGAGGTTTT TATCTTGCAA
27781 TAAAATTGCC TGACGGGCTG ACTCATACGG CAGATGATAG GGTGTCATGC CGGTTTGCCG
27841 GTAAGTGGAC AACATTTTCA TTACACCGTT ATAGTCAGTT TTCTCTAACG TCTGAATATT
27901 ATGCAGCAGT AATTCATTAG ATAAGGATAA TGTGGAAATT TCTTCATCCA TATTATTCTG
27961 TGTCAGTGCC AGTGAAGCAA TGTCGGGGCG TCGTTTATTC AGGTGATATT GAGAATTGTC
28021 AGGATGAAAA TCTTTCGCTT CCCGATATAA TTCTGTTAAA TAAGCCGCTG GTGAAAATAT
28081 GGAAGCAATT GATCCCGGTT TTACAAAACG GTGGGCGCGG CCATAAAACC AACTGTTGTA
28141 ACTATTGTTT AGGGTTGACG GTGTAATATT AAGGTTAGTG ATATTAGCCA GTTGTGGATT
28201 AGCACGGGAC AAAATGCGCA GTTCTTCAAG TTTATTCTGT TTTGATTCCT GATGAGCCTG
28261 TTGATATAAA AAGTCTGTTT CTCGCCACGT CAGAGTTCCA CTTGTCCTAT GACGAAATTC
28321 GCTGAAAGAC ATAAACGAAA TGTTTGTCAA TAATAAAGTA TCACCAGCCT TTTTCTATTT
28381 ATCTTATCTA ACAGTTCATT AACTTTTATC ATATAAATCC TTAAGTTATT GTCAATTTAA
28441 TGATTAATGG TTTTTAGGTG GAGATTATTA TAATCTGATA GGAATATTAT GGTTAATTAA
28501 ATTGATACTG ATTTATCGCT CTATTCTTTC AATAAAAAAT AAAGAACTTC CCTATAATAC
28561 ATGGATTTAA ATAATGAATA CCGTATGTTA AAAATTAAAT TTTAACAAAC TTTCATGAAA
28621 AAATTCAACT CAACAATTGT TTAAATATTT TTAATTGTGT TTGTGCTGTT TGAAAAATGA
28681 ATGACTAATA TTTATCTATG AAAGATTATT TATTGAGGAT GTCTTGCTTG GTTTCAGGGG
28741 GCTACGTTGG AGTCAGATAA ATGTGTGCAA AAAGAAATCC TTAATAAAGT TGCGTAATTA
28801 CAAAAGTTGG TATATCGTGA CAAGAGTGAT AGTAATGTCA CATAATTTAT TGAATACCCG
28861 AACCTCGCAA ATGCGGGGTT TTTCTTCGCA TAATCAAAGA GAAAGCTATG AAAAAAACAC
28921 TGATTACTCT TATTCTCAGT ACCCTTTCTT TTGGTGCTTT GGCACAGCAG GGTGGCTTCG
28981 TTTCCCCGGA CAGCACAGAC TATACTCAGG GTGGATTTAA AGGTCCAACT CCCAACCTGA
29041 CCAGCGTTGC TCAAGCAAAA TCTTTTCGTG ATGATGCGTG GGTTGTTCTG GAAGGAAACA
29101 TTGTTAAACA GGTTGGTCAC GAACTCTATG AATTCGCGGC CGCATAATAC GACTCACTAT
29161 AGGGATCGCT TATTACGGAC TTATCCGGAA AGCTATCTGG AACCCCTGTT ACGCCTGAAT
29221 AAAACAGAAT TCAGGGATAA CAGTGGTTCT GTTTATGTTG ACATTGATGA TAAGCGCTGG
29281 ATGGGTCTGA CGGCCACTCC AACTGACAAA GTTCGTATCG AAGGTGAAGT GGACAAAGAC
29341 TGGAACAGTG TTGAAATTGA TGTCAAAACT ATCCGCATAG TGAAATAACT CAAGCACTTT
29401 GAATATAGCC CCGCACTCGC GGGGTTTTTT GCTTTCTGGG AGTCGGAAGT TTAACCGTAG
29461 TGACGAGGAT CAAAACTAAG TTAACGGCAG TGGTCACTGA TTTGGTGCAT AAGTTATCAA
29521 AAGTTAAAAA TCAAAACTTA TTTTTTATTT AATAGAGGAA TGTCACCCTG TAGGTGAATA
29581 ACGTTGACGG ATGTAAATAT ACAGTATTAT AGTCCTTTGA TATGTTATTA AATTGAAAAA
29641 CCTTTAAACT ATATTCGGGG GAAATTATTA TGTCAGATGT TCGTAATATT ATTAATGTTG
29701 ATAACAATTT TGGTTGTGAA TATAAAGCGG ATTTATTTAA ATAAGTTTTC ATAATTGTGA
29761 TACACCCATT TTTCTCATCC CCGGTTTTTG CTGTTGTAAG GAAGCGGTTT CCATGAAGAT
29821 TTTGACATGG TTAAGCAACT GCCACATAAA TTGGCAGCAG TGGTTTCGTG TCACGGTTTC
29881 ATGCAAGGAT TGCCATAGAC GTTCAATTTT ATTCAACCAC GGGCAATAGG TCGGTAAAAA
29941 GAGAAGATTA AATTTGGGAT TCTTTGCCAG CCAAACCCTG ACCTTCCGGC TCTTATGAAT
30001 GCAATAGTTA TCTAAAATTA ACGTGATGGT TTTGGCATTA ACATATTGAT TGTTAATTTC
30061 ATCTAACAAT TTGATAAATA AATCTGAGTT CTTTCTCAAG CTACCGACAT AAGTGATTTC
30121 TTTCGTTTTC GCGTTGAGGC AATTGGCAAG GTAGTGTTTT TGGTTCTTTC CGGGGGTAAC
30181 AACACGCTTT TGTTGCCCTT TGAAGCACCA GTCTGCACCG ATTTTCGGGT TCAGGTTGAT
30241 GTCCACCTCA TCCTCATAGA AGACCGGGTG TTTCTCTTGA GGCATTGGAT AACGTCTCGC
30301 TGATTTTTGC CATTTTTTCA TCATACTCAG GGTCAGGCAA TTTTACGGTT GGTGCCGCCC
30361 TTCGCCAAAC GATGCCCGTC CGGCAAAAGT AGCGATAGAG GGTACTTTGA GAGAGCGATG
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Fig.2.

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30421  TATTCAGTAG CTCATTGATT TTAAGTGTAA TAAGCTCAAG GCTCCATCGT GAACGGAGAT
30481  AGCCAAAATG TTGTGGCGAG TGCTGTAATA AGAAAGAAAT GACTGTGAAG AGCGGAGCTA
30541  AGTTCCAGAT GGCAGGCCTT CCCGCCGGGA GGCTTTTAAG TCCTTCCAAC CCGTATAATG
30601  TTAACCAATT TACCCAACGA TGAACGGAAG AACGTGAACA GTGAAGCGTT CTGGAAACGT
30661  GAGAAACCGT ACTCCCTTCA TGTAACATCA AGAGCGCGGT GAAGCGACGT GCATAGTCCT
30721  TATCCCGGGT TTTCTGGATA GCTTTTTTCA TCGGACGTCG TTCATTTCGG GGTATTGATG
30781  TTATGATTGG CATGACTCAG TCCATTTTGG GATTTGTTTT GATTTGGCGA TTAATCAGAT
30841  CGCGAAAATC GGACTGAGTT CCCTTCAAGT GATCTACTAT TTTGAAATCT TATTTAATCA
30901  GGAGTCAGCA AATGAGTTAT TCCCCATAAT ACCTGACCAT GTGGTTGTTT ATCCGGGAAA
30961  TGATTCATCT ACCGGTGGTA TGTGGATTCC TTGGTGCGAT AGTCAGAAAG ATATTGACTC
31021  TGGCCATTAT ATCAAAGTTA CTTTCAGTAA AAAGGACGCT GCTGATATTG TGAACTACAT
31081  GTTTCAACAT GGCAGTTATG TTTATTTTAC AGACAGTAGT AAACAATTTA GCAATAAGCA
31141  AATTATGTCT GGTGATTCAG CTAAAGGCAA AGGGGATTAT AAGCTTGAAA TTAAAACAAA
31201  CGGGAACCTT CCACTGATGG TATTGAATAA ATATTGATTC ATTATTATTT ATGGATAAGA
31261  AATTAAGTTT ATATTTCATC TGGTTTCTGC AATTAAGTTT TAAAAATTAA TTCTACTTTT
31321  TTTATGGTTT TATATTTAAT GCCAATCATA TTATTTTTCT TATAATAATT GATAGTTTAT
31381  TTATATAGTA AATAAATTCT GTTGGATGTG ATTATTATTG TGAGACGGTA ATAATTAACA
31441  TAACAGAAAA TTCATGGTTA GGAAATTCAA TCAACTTTTG TCCGGTTTCC TGACCATGAA
31501  GAGCTGTATT TACTGTAGAA CTCGCATTGA TACTGGATTG ATTAGCCGGA CGAGTGTTGG
31561  GTCAGCAGAT AATATGTTGT ATATTGGCTG TGGATTTTTC AGCGAGATGA TAGCTTTGGC
31621  AGTAAAGGCG ATTAATAACC GATAAAACAG AGAGACGGAT TGTGGCCAGG AAAGCAAAAA
31681  AGCCTCACCA TGACGCGTTA TTCAAACATT TTTTAACCCA ACCAGAAACC GCCCGGGAAT
31741  TTTTATCCCT TTATCTGCCG GAAGCGATCC GGTCAGTGTG TGATTTACCA CACTAAAACT
31801  GGAACCGGCA GCTTTGTGGA CAGGCAATTA CGTCAGTTGC ACAGTGATGT GCTGTATTCT
31861  GTCGAGACAA CCCACGGGGA CGGTTACATT TATTGCCTGA TTGAACACCA GTCCACGCCT
31921  GATCCGTTAA TGGCCTGGCG GCTGATGTAT TATTCGCTGT CAGCCATGGC TGCGCATCTG
31981  AAAAAAGGAC ATACTGAACT CCCTTTGGTC GTCCCCCTGC TGTTTTATCA TGGTGAGGTG
32041  AGGCCTTACC CTTACTCAAA TCGATGGCTG GATTGTTTTA CACTCTCTGA ACACGCGGCT
32101  CACCTGTATA ATCAGCCCCT GCCGTTGGTG GATATCAGTG CGCTCAGTGA TGAAGAGATC
32161  CTGACACATA AAAGCATTGC CTTGATGGAG CTGGTACAAA AACATATCCG TTGCCGGGAT
32221  ATGCTGGAGT GGGTTCCCCA ATTGGTGGCG TTGTTGAATG CCGGTTATAA TAGCGCCGAA
32281  CAGCGCCATG TTGTGTTAAG CTATATTTTA CTGAATGGAC ATACGCTGGA TCTCGCCCAG
32341  TTTGTCCATC AACTGACTGA ACAATCTCCG GAGCATGAAA CCATGTTGAT GACTATTGCA
32401  GAACAGCTTG AACAAAAAGG GCGTGAGCAA GGCCGGACAG AAGGCAGAAC AGAAGGCAGA
32461  GCTGAAGGAC GGGAAGAAGG CAAGCTGGAA ACGGCGCGCG CATTATTACG GCATGGTGTC
32521  AGTCTGGACA TCATTGTCAC CAGTACCGGC CTGAGCCGGG AGAAAATTGA AGCGTTAAAG
32581  CATTAAATGG ATACGCTTTT TCACAGCAGG ATATGGTGAC CCCTGTGAGG CCACCGGAAA
32641  ATTTTATTTA CTACGATTTA CGACGGGTTA CTTTAGGAAG CTGAATGAGA CGTCCTTTGT
32701  TATATAACGG TCCCATATCA ATCTTCTCTT TTCCGCGTAC AGGTAAGTAA CCCAAACCTT
32761  CGTGAGCAGC ATTTGCCAAC AGGCCATCAT CCTGATCGCC TGACCAAGAG AAGATCCCGC
32821  CCAATTTCAT TTTGGTTGCA TAAATTCCCT TATGCAGCAC AGTGCGGGGC GTATCCAGTG
32881  AAATCCAGTG ACCACCGTCA GCATTAAAGA GTGCGTCAGC GTCGGTTTCC GTGTCTGTCA
32941  CCAGTTCAAA CTGATTTTTC CCGCGTGCAA TTTCATATTC CGCATCGTAT TGGTTATTCA
33001  GCAGACAGAA GAATTCCGGA GCACCTTTTT CCATCGTGCC CAGTGGCTCT CCTGTTCTGT
33061  TATAGCGGCG CGTTGTCAGA TCAGCACCCA GACATGAACG TCCATAGTTA GCAAATCCGA
33121  GGTGAATTTT CTCCGGTTGT ACACCTTGTG ACAGTAAAAA GCGGATCGCC TCATCTGCCG
33181  AGTAATCCAT GTCCCGATCA GGATTGGGCG GAGGAGGGTT ATCGCCGTCA TATTCATATC
33241  TGGGGGGATA CAGGTTAGTA TGGTGACCGA TGTATTCTGC CCAACCGGTA CCAAAGAAGT
33301  CGTAGGTCAT CACAAAGATA TTGTCTAAAT AAGGTGCGAT TTCTTTGAAG CTGGACTTCT
33361  CCATTTTGGC AACGACGGCG CTACAGGCTA TCGTGATTTC TTTACGGGCC CGGGTTCCAA
33421  AGGCGATGTT CAGTGCTTCA CGCAGCTCTT TCACTAACAA AACATAGTTT GGGCCATCAT
33481  GTTCCGGGTC GAATTCATTA CCTTCTTCAC CTGTGGCGCC GGGGTATTCC CAGTCGATAT
33541  CCACCGCAGT AAACATGGGA AAACGCCGGG AAGAAGTCGA CGATGCTACT CACAAATGTA
33601  GCACGTTGCT CAGGATCTTT GGCCATCACA GAGAAATACC CTGACATACT CCAGCCGCCG
33661  ATACTGAATG CGAGTTCCAG CTTATGCCCT GCCTGTTTTG CTCGCGCTTT CAGATTACGC
33721  AATCCCCCCA GTAAACCGGA GGCTGCATCC TGATTGTAAT ATTGCAAGAA ATTCTTCGGG
33781  CTGGCATCAC GGCGCTGATC CGCGTCCAGA CCGACATTGC GTGTGGTGCC TAAATCACCA
33841  TAAGGATCAA CGGGTACAAT ATGGCCTAAT GTAATAGGGG CAATCTGGCC ACTGCTGGCT
33901  TCTGCTTGCC GGTTCCACCC GTCAACAACC TCATTAATCC GTTCGGATAA CTTGCCTTTG
33961  TCACCGTTGA CGGCCATAAA ACTGAAAATC AGGCGGTCGT AGGCGGTAGG CGGGATTTTT
34021  TCCAGATCAA AACCACGGCC GGGGGCATCG TCGCTGGTCA GCGCAGTGTT ATCCTGGGTT
34081  TCTGGCGACA AACGCGCATC ATACTGGCAC CAGTCAGTAA TATAGGCAGA GACTTTAGGC
34141  AGCGGTTCTG TATTTTCCGG ATCAACTTCA TATTCGTTGT ACAGGGACTT GGCAACACGT
34201  GCTGAAGAAT AACTCAAAGG AGTTCCGCTG CCGTCAGGTT TATATCCCAC CTTCTGATAG
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Fig.2.

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34261  GTTTCTTCTG TGAGTGCATC ATATTGCAAT ACCTCGGTTT TTTCTCCCGG CGGTACATCA
34321  GGCGTATTGG GGTTACCGTG ATCGGCAATT TCTTCCGGTG TCGCCTCACG GACATATTGC
34381  CAGGCATTCT CATAAACCGG TAAATCAGGT GAAATATTGC GGTCGGGAAT ATGCCAGCGT
34441  TCAACCCAGC CGATGTTTTT AAAAACCGCG CTATCATAAA TGACATACCA GGTTTGACCA
34501  CCAGATTGAT TCTGCCAGGC AACCAGAGAT GCGCCTACTT CGCTGCTGGC GTCAGACATC
34561  GCTTTAATTG AAGGGTATCG ATAAACATTT TGAGACATAA TTTCACTTCC GGCCCCGTTA
34621  TATTCCGGGG CCGGCTCCTG ATATCAGTTA GAATTGTCTT GTTTTAATTG ATGTTTATTC
34681  AGACGGCTAC GAACCTGCTG GCTGAACTCA TTACTTCCGC CACTCACATC ACGCGCGGTA
34741  TAACGCAGAT GGAGGATAAT ATCGCTCAGC GACTCCAGCA GCTGATCCTG ATCGGAACCG
34801  AATTCCAACT TCCACTGTGA AATGGCGCCT GTCCCTTCAA AAGGCAGGAA AAGTTCATCA
34861  TCAAAATTGA GCCTGAACAT GCCGCTGTCT TCCATGGCCG TTGAAATCAC CACACCTTGA
34921  TTAGCCTGTA CGTTCAGCAA AACGTTTTCG GGTTTGGTGT ATTCCAAGGG GTTAAGCAAA
34981  TAATCGATAG TTTTTAAGTC AGCAGTACTG TAAAGCGTAT TGCTGAGTTG TACCAGTGAA
35041  GCCCGTACAT CTTCATAAGG CCCCAGCAAT GCGGGCAATG ACAGCGCTAC GGTTTTTATA
35101  CGCCGATCAG CGTGGGTCGG ATAATCGCGC AAGAACATTT CGGCGCTCAG TAAGAAAGTG
35161  AATGAACCCG TACTCTTGCC AATTTCCCAC TGTGATGATG TCAGTAATGA TTTTACCGAT
35221  ATGGTTTTTA TGATCTCCAG ACGTCTGGTG TTATGTTGCA AATACGCCTG ATCCATCCGT
35281  TGTAAGGCTA ATTTCAGATG TTCTCCGACC AGCAGCCCCT GATAAAGATC ATTCCAGAGA
35341  CCACTTTGGA CGAAATTCAT ATCATACTGA CCTGTTTCGT ACTGCCAGGA GGCTTCGGCC
35401  AGTAAACAGA GGGAATTAAC CGCATCATAG GCTTGCAGGT AAAGCCGGAG ATTTGGCTGA
35461  TCATCCACAT GTATAACGCA TCATTGGTAN ANTTGTTCNN NNNNNNNNNN NNNNNNNNNC
35521  CCGAAGCATA CCGCCAAGAC CATCCCCCCG ACGGCCAGAC CGAAAATATT GGGAACCATA
35581  TCCGCCACAG CGGCCGCAGT GGCGGCTGAC TGGGCAGCGA TCACACCTTC AGCCGCTCTT
35641  GATTGTAATG CGATAACTTC CTGCTCGGTG ATGGAGATGT TTTCATCATA GAGCGATTTA
35701  TAGTGTTGCT GGCGCTCCTG AGCGGCCCGT CGGCTGATGG TCAGTGCATC CAATGAAGCC
35761  TGTTGCATGT CAATCGCTTG CTGTTGCAGA TTGCGGGTAA AGCTGTACAG CCCCAGTTGC
35821  TGCTGCATAC GGAAGTGTTC AAAATCGGTA TTGTCTTTTT TCTCCAGCAA ACTCAGTAAC
35881  GTGCTGCCGT ACTGAATCAG CGTTTCTGCG GCCTCTTTTG CCCGGCTCAT GATCGGGGTG
35941  AAACGATAAT TCGGGATTGC CCGGCGTTTC ATGCCCGCCA TACGATTAGC CACAACACGC
36001  TGGTAACGCT GCCTGAGCAG ATCTTGCGGG CTGATGGGTT CATCGTATAA TCCGGCCGGA
36061  AACTCTTTAC CATCCAAGGT CAGGTTATGA CGTAAGTTAT ATAGACGCTG ATCCAACATT
36121  TGCCACAGTT TGAGATATTC CGTATCAACA GGTTTGACAA ATAAATCAGA CGGTGCGGCA
36181  GAGACGGATG TATCATATGT CACAGGCAGA AGTGGCACGT TGCTGACAGT AAGCATTAAC
36241  TCCTGTGCCC GTGCTTCACT GTTTTCATAC AGAGCCACAT CTTGCAGCGT ACGGGGTTGC
36301  CAGTTTGCCG CGAGCAGAAT ATCAGGGCTG GTACCCAGTA ACATATTGAC GGAGTCATAG
36361  ATCTGCTTGG CGACAGTACG TGCACTGGAT GTCAGCTTAC GGTATTCCAT GTCTCCCTGA
36421  TCTAACAGAT TCTTGACATA GAAACGGAAT ATTGCTTTCC GGTAGTGAAT GGGTTCACTG
36481  GCTGCAATGG CATCCGGATC GGTTGGTTCA ATTAACATCC GGTACACGGT GGGTGGAGGA
36541  TCAATAATTG GCCGTGAATT CCAGTAACGC GGTTTACCTT GGTTGCTGGC CTGAACAAGT
36601  TCATCTTCCA GCGGATTAAA AATATAGTGC AGCCATTCGG TGGCCTCTTT TAATCGTTGT
36661  TCTATATTCA GTCGCCACGC GACCAGAAAT GGCATATGGA AAAACAGTTC CCAGAAATAG
36721  ATCCCATTTG CGCCATTTAA ATCAATCGGC GTAGGGAATG AACCGGGTAT AGGCTGTTCG
36781  GTAATAAGCT GTGTATTCCA GCTCAGTACC TGCGGGATAC CCTGACTGGC AATGGCGATC
36841  AGTTTTTTTG CAAACAGTGT ATTAAGGCGA ATGTTTTGTG GCGCGTTATC AGTTTCATCT
36901  GCGGGGAAGG AAAGGAATTG CACCTGATCC TGTTCATTGA GTTTAATCAG TTCGCGAATA
36961  TGCATACCGA TTCTGAACTC TTGAGTACAG CTGGCACTTT CATTGCCAAC ACCACCTTTG
37021  GGCTTAAAGA GAAGTTCGGC TTTCAGGGTG ATTCGATTAT CCGACCCCAG CTTGATTGAT
37081  GGATAGGTTA AATCAAGAAC TTTTTCGCTC AGTACCAGTG GTTGTTCATC CAAGACAGTA
37141  TTATCGTGCA TCAGCCGGAA AGAACCGTTG TAATATTGAT GATCTTCTAT CGCACCAAAC
37201  TTAAAGTCAG ATTGAGCGAC AATCTCCAGT GTGTCATCAG TGCCATGAAC AAAATTGACA
37261  ATCAGTTTGA TACTGTCTTT GCCGAAATCA GGGTTCATTC CGGTTTGGAT TCTCCGGCAA
37321  TAGGAAAGCG TTCTTCCCGG GTTGCCGGAT AGAGCACCAT AGTACGGTAA TCGATAGGAT
37381  TGCCTTAAGG CATCCTTGTG TTCACGTGAG TAATACCAGA CCAGGTTGCC GACATATTTT
37441  CCTTTTCGTC CATCAGCATA TTGGTCATCC GGCAAATCAG TAATTTCTAC CAGCAGTGTA
37501  TCGCAGACAT AACCGAAGGC TTCGTCATAA TCATAATCCT TACCTTTCTT ATCTGTCCCC
37561  TGAAGACGGA CAAACGGAAC CAGAGCCAGA AACGGGTTAT GCGGGTCTTG CTGTATATCC
37621  ATCACAGCAA CCATCTGGGC CATCCGGTAT TGCAGATGTC TTCGCGCAGA ATGGTGGGTG
37681  TACTCCAGCT GCCATCATAT TTGGCATAAG CGATTTTGAT CCGGTCAGGA ACGGTGTGGG
37741  AGGAACCCAA TCACCCGCAC TAGGCTCAAC GTTTTGGTTA TGCAGTGATA ACGCAGTTGT
37801  ATCTTTAGTT TCAGACTGTT CTTCAACTTC CGTCCAGGCA ATATACAGGC GATTATTCAG
37861  GAAAATGGGG CGTATCAAAT TGGGGTCTAC GCTGCCCAAT GGCAGGTCAA TAGGTTTCCA
37921  CTCGCTCCAG GCATTGGGAG ATAACGCATC GGTATCAGGA TGGCGTATCG AAAGATTCAG
37981  TGAACGCCAG TAATATTGGT ATGGCTGTGT ACGGGTACGT CCGACAAAGA AGAACTTATC
38041  GCGTTTGATG TTAACACCAT CTTCATAACC TGCGATAACT TTCAGGTTAC TGACATCTTC
```

Fig.2.

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38101  AAAATTATTC AGATAACCGA GCACCGCTTG TTGTACAGAA TCTTCGGTAA TTTTTCCCTG
38161  ATTAAGGGCA CTTTCCAGTT GGAAGAAGAA TTCTGTTTTA TTCAGGCGTA ACAGGGGTTC
38221  CAGATAGCTT TCCGGATAAG TCCGTAATAA GCGATCCC
```
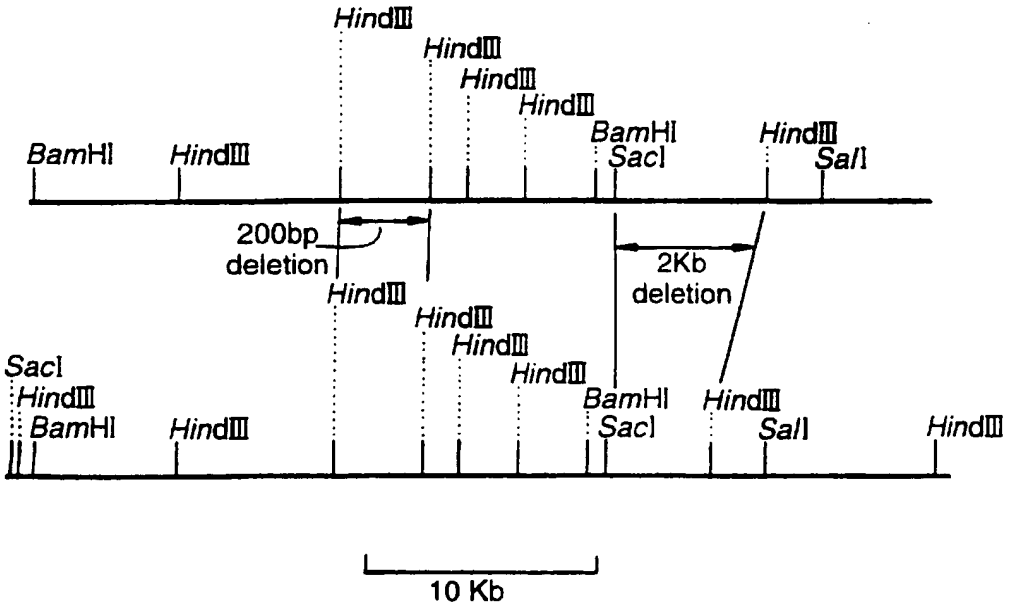

N=unspecified base

Fig.3.



INTERNATIONAL SEARCH REPORT

rnational Application No
PCT/GB 97/02284

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01N63/02 A01N63/00 C12N1/20 C07K14/24 //(A01N63/02, 63:02,63:00),(A01N63/00,63:00)

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 00647 A (COMMW SCIENT IND RES ORG ;SMIGIELSKI ADAM JOSEPH (AU); AKHURST RAY) 5 January 1995 cited in the application	1,5,11, 13, 18-21, 24-26, 29,30,32
Y	see page 1, line 3 - line 29; claims 10-13	3,4, 6-10,12, 14,27, 28,31

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of theinternational search	Date of mailing of the international search report
17 December 1997	14/01/1998

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PCT/GB 97/02284

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMICAL ABSTRACTS, vol. 118, no. 1, 4 January 1993 Columbus, Ohio, US; abstract no. 3550, YAMANAKA, SATOSHI ET AL: "Biochemical and physiological characteristics of Xenorhabdus species, symbiotically associated with entomopathogenic nematodes including Steinernema kushidai and their pathogenicity against Spodoptera litura (Lepidoptera: Noctuidae)" XP002048914 see abstract & ARCH. MICROBIOL. (1992), 158(6), 387-93 CODEN: AMICCW;ISSN: 0302-8933, 1992, ---	3,6
Y	DATABASE DISSABS STN-International / UMI Company STN-AN 96:33246, DISSABS order no. AAI9608671 , 1995 DAVID JOSEPH BOWEN : "Characterization of a High Molecular Weight Insecticidal Protein Complex Produced by the Entomopathogenic Bacterium Photorhabdus luminescens (Nematodes, Biological Control)" XP002048915 see abstract & DISSERTATION ABSTRACTS JOURNAL INTERNATIONAL , vol. 57, no. 1B, 1995, page 93 ---	4,12,14
Y	EP 0 238 441 A (CIBA GEIGY AG) 23 September 1987 see page 1 - page 2 see page 4, paragraph 3 - page 5, paragraph 2; claims 10,12,22,36,37 ---	7-10,27, 28,31
X	WO 84 01775 A (COMMW SCIENT IND RES ORG ;BIOTECH AUSTRALIA PTY LTD (AU)) 10 May 1984 cited in the application see page 1 - page 3, line 10 see page 4, line 24 - line 28 see page 4, line 36 - page 5, line 3 see page 14, line 17 - line 29 see claims 26,27 --- -/--	1,4,5, 11,13

INTERNATIONAL SEARCH REPORT

national Application No
PCT/GB 97/02284

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	H.MATSUI ET AL. : "Nucleotide sequences of genes encoding 32 KDa and 70 kDa polypeptides in mba region of the virulence plasmid, pKDSC50, of Salmonella choleraesuis " NUCLEIC ACIDS RESEARCH , vol. 18, no. 8, 1990, pages 2181-2, XP002050055 see the whole document ---	21-25
X	F.BINDER ET AL.: "Cyclodextrin-glycosyltransferase from Klebsiella pneumoniae M5a1: cloning nucleotide sequence and expression" GENE, vol. 47, 1986, pages 269-77, XP002050056 see page 269, the summary see page 270, right-hand column, last paragraph - page 271, right-hand column, paragraph 1 see fig. 3 bp 2641-2809 ---	21-25
P,X	US 5 616 318 A (DUDNEY RALPH A) 1 April 1997 see column 1, line 65 - column 2, line 52 see column 5, line 3 - line 4 ---	1,4-6, 11,13
T	WO 97 17432 A (WISCONSIN ALUMNI RES FOUND) 15 May 1997 see page 2, line 31 - page 3, line 23 see page 5, line 1 - line 16 see page 8, line 23 - line 33 see page 9, line 41 - page 11, line 14 see page 17, line 1 - line 21 -----	1-32

INTERNATIONAL SEARCH REPORT

national Application No
PCT/GB 97/02284

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9500647 A	05-01-95	AU 675335 B	30-01-97
		AU 6991694 A	17-01-95
		EP 0705340 A	10-04-96
		JP 9500264 T	14-01-97
EP 0238441 A	23-09-87	GB 2188049 A	23-09-87
		AU 608508 B	11-04-91
		AU 6999287 A	17-09-87
		BG 46006 A	15-09-89
		BG 46752 A	15-02-90
		BR 8701162 A	12-01-88
		DE 3788077 D	16-12-93
		DK 128687 A	16-09-87
		EG 18869 A	28-02-94
		ES 2059404 T	16-11-94
		IE 59456 B	23-02-94
		JP 62224295 A	02-10-87
WO 8401775 A	10-05-84	AU 558287 B	22-01-87
		CA 1214130 A	18-11-86
		EP 0126092 A	28-11-84
		US 4672130 A	09-06-87
US 5616318 A	01-04-97	NONE	
WO 9717432 A	15-05-97	AU 1050997 A	29-05-97
		CA 2209659 A	15-05-97
		EP 0797659 A	01-10-97